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IOWA	
State University of Iowa	March 29, 1954
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Fitzsimons Army Hospital	February 26, 1954
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Cornell University	November 14, 1953

Comparison of 17-Ketosteroid and Uropepsin Data on 69 Healthy Adult Males.* (20994)

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During the past few years a number of investigators(1,2) have suggested that uropepsin excretion is an indicator of adrenal cortical activity. Others have questioned this assumption. Having collected considerable data on uropepsin and 17-ketosteroid excretion in the same individuals during the course of another type of study, we decided to analyze our data to see whether any correlation exists between these two measures. It is recognized generally that the larger part of the urinary 17-ketosteroid excretion is derived from the adrenal cortex, with the lesser part coming from the gonads(3). Many workers have shown that 17-ketosteroid excretion in normal individuals is increased following ACTH administration(3,4). The complete relationship between cortiscosteroids and 17-ketosteroids is not yet known, but there is evidence which indicates that normal C_{21}-11-desoxy and C_{21}-11-oxygenated hormones of the adrenal cortex contribute to the titer of urinary 17-ketosteroids(4). In the opinion of investigators who have made an extensive study of the methods of extraction and assay of the urinary steroids related to the adrenal cortex, the methods for the urinary corticosteroids do not give dependable, quantitative data(5), whereas those for the urinary 17-ketosteroids are generally reliable(6). As a consequence, in the present study the measurement of 17-ketosteroid excretion was selected as the index of adrenal cortical activity.

During the course of an investigation of certain biochemical factors in healthy male sex offenders data were collected on the 17-ketosteroid and corresponding uropepsin levels

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TABLE I. Urinary Excretion of 17-Ketosteroids and of Uropepsin.

Age, yr	No. of persons	Avg day to night ratios	No. of persons	Avg excretion, mg/24 hr	Median values mg/24 hr	Chi square	Probability level
17-Ketosteroids							
17-35			46	10.97	10.4	5.67	.017*
36-58			23	8.24	7.1		
17-30	35	1.30	37	10.68	10.4		
31-40	14	1.69	16	10.55	9.4	4.69	.096
41-58	14	1.30	16	8.12	6.7		
Uropepsin			West, units/hr		West, u/hr		
17-35			46	36.13	29.3	.09	.800*
36-58			23	42.24	26.5		
17-30	35	1.38	37	37.26	29.5		
31-40	14	1.39	16	41.00	36.5	.57	.720
41-58	14	1.47	16	37.44	24.5		

* Difference between age groups may be expected to be due to chance 17 and 800 times, respectively, in 1000 samples.

of 69 individuals. Since administration of ACTH increases the excretion of both 17-ketosteroids(3) and uropepsin(2) in healthy individuals it seems logical to assume that a correlation might exist between the excretion levels of these two entities without the administration of ACTH. Such evidence would strengthen the belief that the determination of uropepsin can be used as a measure of adrenal cortical activity. The purpose of this paper is to present the results of a comparison of these data.

Subjects. The present study utilized 2 groups of male subjects. The first of these was composed of 55 healthy sex offenders who had been committed to Metropolitan State Hospital for a period of observation and treatment under the California Sex Psychopath Act. All persons used for this study had been given routine physical examinations including chest x-rays by the hospital staff. Those who showed evidence of organic disease were excluded. A further check was made by determination of white blood counts and sedimentation rates immediately prior to the collection of the urine samples to be used for the 17-ketosteroid and uropepsin determinations. The second group was composed of 14 male medical students and research workers at the University of California at Los Angeles. For the purpose of comparing the 17-ketosteroid excretion with uropepsin output these two groups of subjects were combined.

Methods. One to 3 pairs of consecutive 12-hour urine specimens were collected on each subject. The first morning specimen of urine was discarded. The day collection was begun immediately thereafter and continued for 12 hours. Collection of the night specimen was then begun and continued through the first morning specimen voided. Care was taken to protect these specimens from light and to keep them refrigerated. Steroid and uropepsin determinations were run on the same specimen; the uropepsin determinations were done within 36 hours of the start of collection and the steroid extraction within 50 hours. 17-ketosteroid determinations were made by a method of hydrolysis and extraction perfected in this laboratory(7). In this extraction process 6 N sulfuric acid is used in place of concentrated hydrochloric acid for the hydrolysis of the conjugates. The assay method used is a modification(7) of the Callow absolute alcohol technic for the Zimmerman determination. Uropepsin determinations were made according to the method of West *et al.*(8). The endpoint of this method is the clotting of milk by the urinary enzyme at pH 4.9. West's method is rapid, reproducible, and extremely sensitive. The results reported in this paper in units per hour are 1/24th of the total 24-hour excretion per person. Output of uropepsin per hour for normal individuals falls between 10 and 40 West units.

Results. In order to determine the relationship between 17-ketosteroid and uropepsin excretion, comparisons were made of the 24-

hour excretion values and of the day to night ratios, respectively, of the two measures. In all instances, data for both measures were obtained from the same urine specimen. It should be noted that the values for both measures cover a 24-hour period for the absolute excretion values and a 12-hour period for the calculation of the day to night ratios (essentially waking-sleeping ratios). Urine samples collected over much shorter periods of time might give erroneous relationships between 2 or more biochemical measures because of different rates of reaction during the interval between the initiation of stress and the collection of the specimen.

a. 24-Hour excretion values. The average and median values of the data obtained in this study are given in Table I. In agreement with the findings of others the 17-ketosteroid data(3,9) are seen to vary significantly with age, whereas the uropepsin values(10) show no such variation. Because of this variation in 17-ketosteroids with age, the data obtained were originally divided into two categories; one for individuals 17 to 35 with a median age of 25, the other, 36 to 58 with a median age of 43. Further analysis of the data, however, disclosed that whereas this division into two age groups shows the only statistically significant difference in 17-ketosteroid excretion values with age, it appears to mask the relationship between 17-ketosteroid and uropepsin excretion. This could be due either to the lack of variation in uropepsin excretion with age, or to the influence on uropepsin excretion of factors which have no special bearing on 17-ketosteroid excretion. Accordingly, the data were divided into 3 age groups for comparison of these measures. It seems probable that the reduction in 17-ketosteroid excretion with age is due to both decreased adrenal cortical activity and gonadal activity. Pincus(9) has reported that while the difference he found between the 17-ketosteroid excretion of young and old men might be attributed to testis precursors, the quantitative relationship (young and old men *vs* true eunuchs) is not entirely reconcilable with this explanation. He suggests that either an adrenal cortical 17-ketosteroid precursor is produced in lesser amounts in older men,

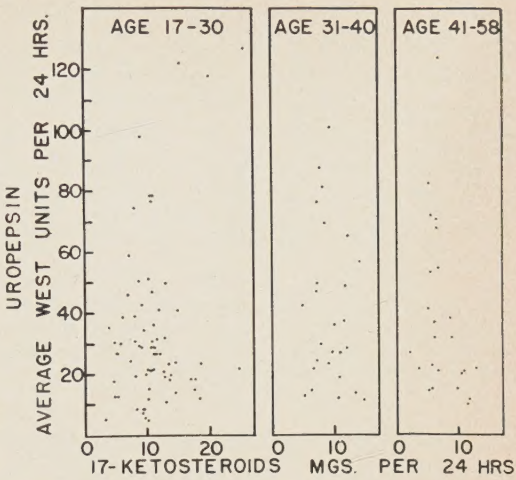

FIG. 1. Comparison of 17-ketosteroid and uropepsin excretion levels in 3 age groups of healthy males. Both determinations were made on the same 24-hr urine specimen in each case.

or that these precursors are differently metabolized in the two age groups. Several comparisons have been made of the 24-hour data obtained. Scattergrams (Fig. 1) of the individual 17-ketosteroid and uropepsin excretion values (true pairs) show the relationships of these values. The correlations for these data are given in Table II A. It will be noted that a significant correlation is obtained for each of the extreme groups, a low positive one for the youngest group and a higher negative one for the oldest group. The intermediate group shows a transitional state.

b. Day to night ratios. In agreement with other investigators(3,11) we have obtained day to night variations in 17-ketosteroid levels. Using the West milk-clotting method,

TABLE II. Pearson Correlation Coefficients for 17-Ketosteroid and Uropepsin Values.

	No. of persons	No. of values	r found	Probability level
A. 24-hr values				
17-30 yr group	37	69	+.261	.032
31-40	16	28	—.101	.582
41-58	16	26	—.469	.012*
B. Day to night ratios				
17-30 yr group	35	67	+.500	<.001*
31-40	14	26	+.828	<.001*
41-58	14	24	—.162	.453

* Difference between age groups may be expected to be due to chance 12 times and less than once, respectively, in 1000 samples.

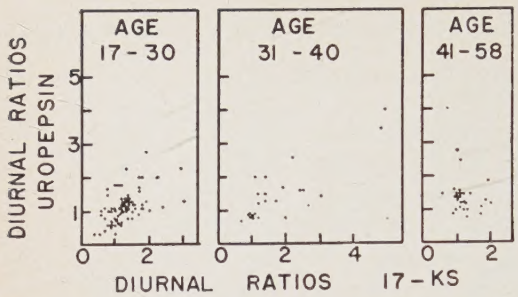

FIG. 2. Comparison of diurnal variations in 17-ketosteroid excretion with corresponding diurnal variations in uropepsin levels. Both determinations were made on the same 12-hr urine specimen in each case.

we have also found day to night variations in uropepsin output. This is in contrast with the findings of Mirsky *et al.*(12) who used a hemoglobin digestion method for uropepsin determinations.

Day to night ratios for 17-ketosteroid excretion are believed to reflect the difference in the amount of stress to which the individual is subjected during the waking period as compared with the sleeping period(3). For most individuals this ratio is greater than unity. Some show the reverse picture. Since no special stress situations were employed during the collection of these data, the day to night ratios may reflect to some extent the normal diurnal variations in metabolism.

The day to night ratios were divided into three groups according to the age of the subject, the age limits being the same as those used for the 24-hour values. When the ratios for the youngest group are compared (Table II B), a significant positive correlation is obtained. An even higher positive correlation is obtained for the intermediate group. The ratios for the oldest age group show a negative relationship of no significance. Distribution of the day to night ratios for these 2 measures is shown in Fig. 2.

Discussion. The low positive correlation of the 24-hour excretion levels of 17-ketosteroids and uropepsin for the 17-30-year group may indicate that a reactive adrenal cortex can exert a measurable influence on the level of uropepsin excretion. This suggestion is substantiated by the high positive correlations of the day to night ratios for these two measures for the two age groups, 17-30 and 31-40. If this hypothesis is correct, then the high negative correlation of the 24-hour values for the oldest group (41-58 years) may mean that the lessened activity of the adrenal cortex (indicated by the declining 17-ketosteroid excretion) is no longer effective in affecting the level of uropepsin secretion, and that the sustained or increasing uropepsin levels in these persons are primarily under some other influence. An alternative explanation for this negative correlation might be that a decline occurs in the secretion of adrenal cortical 17-ketosteroid precursors by the 41-58-year group without a decline in the adrenal cortical factor affecting gastric secretory activity. In a study of stress responsivity in normal, healthy men, Pincus (9) found that the total adrenocortical responsivity of the older group (median age 77) was generally slightly, though not significantly, lower than that of the younger group (median age 32).

Attention should be called to the data for the 31-40-year group. The 24-hour values for the two measures show no relationship, a finding which is in contrast with the significant positive correlation of the day to night ratios. The diurnal excretion of both 17-ketosteroids and uropepsin is very high, but *only in relation to the nocturnal excretion* since the 24-hour excretion level of both of these measures is average.

There appear to be 2 pathways by which stress of various types may influence gastric secretory activity. Stimuli from the cerebral cortex via the hypothalamus may be relayed to the stomach either by neural pathways to the vagal centers, or by hormonal pathways through the pituitary and adrenal cortical glands(13). Some workers(14) have found that uropepsin excretion does not follow gastric pepsin secretion, whereas others(15) have obtained evidence that they are directly related, at least under basal conditions. It seems reasonable to assume that some relationship does exist since the administration of ACTH or cortisone increases uropepsin excretion, and may cause reactivation or perforation of peptic ulcers(13). There is considerable evidence that stress as well as exogenous ACTH increases the secretion of the

TABLE III. Data on Patient M.P., A Suspected Hypopituitary Case (Age 29). 7 samples.

17-KS, mg/12 hr*	Uropepsin, units/hr	Sedimentation rate†	Eosinophile count	Leucocyte count
—	11	3.5	1606	9600
—	26	2.0	1573	9300
—	33	3.0	1507	10300
—	49	6.0	1355	8800
—	60	—	—	—
.00	37	—	—	—
.10	20	—	—	—

* A value less than 0.50 mg is questionable. This probably does not represent ketosteroid material.

† Normal range: 0-10.

adrenal cortex(3,13). The data presented here appear to indicate that uropepsin excretion in young men is measurably influenced by stimuli carried over hormonal pathways via the adrenal cortex. In older men the negative correlation may mean a difference in the production of the adrenal cortical factors which affect uropepsin and 17-ketosteroid excretion, or it may mean that stimuli affecting the production of uropepsin are carried primarily over neural pathways.

Special mention might be made of one young subject who was not included in the over-all study (Table III). This individual showed no 17-ketosteroid excretion although his uropepsin excretion ranged from 11 to 60 West units per hour. Four abnormally high eosinophile counts, taken on different days, substantiated the indication of adrenal cortical insufficiency. This case is mentioned since it shows the possibility of a normal uropepsin excretion in the complete absence of 17-ketosteroid output.

Summary. 24-hour uropepsin excretion levels were found to show a low positive correlation with 24-hour 17-ketosteroid excretion levels in healthy young men. To an even greater degree this is true of the day to night excretion ratios of these two biochemical measures. Since 17-ketosteroid excretion is believed to reflect adrenal cortical activity, the findings point to the possibility that adrenal cortical activity may exert a controlling effect on uropepsin excretion in young men. Older men were found to show a negative correlation in the 24-hour excretion levels of these two measures with a trend toward a similar relationship between the day to night ratios.

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Effect Upon Inflammation of Topical Treatment with Trypsin.* (20995)

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Several recent experimental studies deal with the effects of various enzyme-preparations upon inflamed tissue. Using the "granuloma-pouch" technic(1,2) as a test object, it has been noted that a fully developed granulomatous membrane is extraordinarily resistant both to trypsin(3) and to pepsin-containing fresh gastric juice(4). Consequently, the presence of inflamed tissue proved to afford an effective barrier against tissue-damage by these enzyme preparations. This could not have been solely a dilution effect with exudate accumulated in the pouch, since the contents of the latter had been evacuated prior to the introduction of the enzyme solutions. On the other hand, Seifter(5) reported that, if hyaluronidase is introduced into connective-tissue, simultaneously with a chemical irritant, (*e.g.*, paraphenylenediamine or allylisothiocyanate) the damage caused by the latter is actually aggravated, because the inflammatory response tends to spread over a greater area.

It has been our experience that the local protective value of irritated connective-tissue is essentially different during the acute stage, just after the eliciting injury is inflicted ("local alarm reaction") and during the more chronic stage, when the organization of a distinct granulomatous membrane has been completed ("local stage of resistance")(1). The experiments to be described in this paper have therefore been undertaken to compare the effect of trypsin upon normal, acutely inflamed and more chronically inflamed connective-tissue.

Materials and technics. Forty female Sprague-Dawley rats, weighing 130-150 g (average 138 g), were subdivided into 4 groups, as outlined in Table I. In all these animals, a symmetrical connective-tissue compartment was delimited by the insufflation of 25 ml of air, under the dorsal skin, between the shoulder blades. In Groups I, II, and III, this was immediately followed (without withdrawing the needle) by the injection into the cavity so produced, of 0.5 ml of a 1% croton-oil solution in corn oil. The details of this procedure, which leads to the formation of a typical granuloma-pouch, have been described elsewhere(2). In Group IV, air was insufflated in the same manner, but this was not followed by the injection of croton oil. Under these conditions, the connective-tissue lining the air-sac shows no inflammatory reaction and retains its essentially normal histologic structure. As a trypsin preparation, we used Armour's "Tryptar", in the form of a solution containing 5 mg/ml, freshly made up in Sorensen's buffer solution.† Two ml of this trypsin preparation was introduced into the airspace of each animal, in Groups II and IV immediately after the pouches were prepared, and in Group III 5 days later, when the croton oil had already transformed the lining into a well-organized granulomatous membrane.

TABLE I. Effect upon Inflammation Produced by Croton Oil of Topical Treatment with Trypsin.

Group	Treatment	Necrosis	
		Incidence (%)	Extent (mm^2)
I	Croton oil	0	0
II	Croton oil & trypsin simultaneously	100	75 ± 3.2
III	Croton oil & trypsin 5 days later	0	0
IV	Trypsin	20	5 ± .1

All experimental animals were killed (simultaneously with the controls of Group I), 48 hours after the trypsin injection. At that time, the entire dorsal skin was removed, flattened on a board, and the parchment-like dark-brown necrotic areas were measured planimetrically, as described in an earlier paper(2).

* This work was supported (in part) by the Medical Research and Development Board, Office of the Surgeon General, Department of the Army.

† Buffered at pH 7.1 prepared by dissolving anhydrous dibasic sodium phosphate 6.6 mg/cc anhydrous monobasic potassium phosphate 2.7 mg/cc.

Results. As outlined in Table I, this, comparatively dilute, croton-oil solution caused no skin-necrosis in any of the controls (Group I), which were not treated with trypsin. On the other hand, the rats, in which croton oil and trypsin were administered simultaneously, all showed necrosis affecting considerable areas (75 mm^2 with a standard error of 3.2).

The rats in which the croton oil was administered 5 days before the trypsin (Group III), on the other hand, did not differ visibly from the controls, in that no skin-necrosis occurred in any of them. Under these conditions the trypsin also failed to induce any obvious change in the intensity of the inflammatory reaction itself. However, since this point was not relevant to the present study, we made no effort to appraise it exactly in quantitative terms. In any event more chronic experiments will have to be performed to assess the effect of the enzyme upon the later course of inflammation.

Among the animals receiving trypsin, without croton oil (Group IV), only 20% showed minute patches of necrosis (5 ± 0.1).

From these observations, it is evident that, in this experimental arrangement, trypsin given simultaneously with croton oil results in widespread tissue damage. This is presumably because here the irritant did not yet lead to the formation of a protective granulomatous barricade, and the damaging effect of the enzyme was merely superimposed upon that of the croton oil. It may be added that, in this group, the edematous and hemorrhagic inflammatory mass greatly exceeded the limits of the original air-sac, spreading towards the anterior surface of the chest. Apparently, the trypsin actually enhanced the dissemination of croton oil throughout the tissues. In view of the interesting observations of Innerfield and coworkers(6), who were able to produce antiphlogistic effects by intravenous and intramuscular trypsin treatment, this inverse effect is especially noteworthy. It calls attention to the fact that, at least under certain circumstances, trypsin can augment the tissue-damage produced by an irritant, conducive to an inflammatory response.

Conversely, after croton oil has already induced a well-formed granuloma, the tissue-damaging action of the enzyme is virtually abolished, and the adjacent tissues are protected. The mechanism of this protection is not yet known. Since, in the present experimental series, the trypsin was introduced on the fifth day, at a time when the pouch still contained only a few drops of exudate, it is unlikely that the overwhelmingly large dose of the concentrated trypsin solution (10 mg in 2 ml) could have been diluted beyond an effective concentration. This possibility is also rendered improbable by the experiments mentioned in the first paragraph of this paper, in which solutions of trypsin and pepsin had been introduced into fully developed granuloma-pouches, from which the exudate was completely removed by aspiration and yet, the phenomenon of protection remained evident. It is possible, on the other hand, that there occurs a considerable concentration of anti-enzyme factors in the granuloma-pouch, or in the few drops of exudate within the cavity, and such chemical inactivation might well be the basis of the protective effect here described. In man, approximately the same amount of trypsin is now given intravenously for the suppression of inflammatory tissue. Since here, activity is retained even after dilution with the entire blood-volume of the patient(6), such a concentration of anti-tryptic power in the granuloma-pouch is indeed rather improbable.

Finally, we had to consider the possibility that fatty acids, liberated from the oil, might have caused the inactivation of trypsin in the 5-day series, either because of their unsaturation or because of a lowering of the pH. To exclude this possibility and, at the same time, to show that a granuloma produced by an agent other than croton oil can also give protection, we performed a small additional experiment, in which carrageenin (kindly supplied by the Seaplant Chemical Corp.), was used as an irritant. The technics for the production of a granuloma-pouch and the experimental animals were similar to those employed in the main experimental series, except that here, in 10 rats, a granuloma-pouch was produced by the injection of 4 ml of a 1% aqueous solution of carrageenin, while 10 other rats acted as controls. On the fifth

day, the exudate which had accumulated in the carrageenin-treated animals was emptied and 4 ml of the above-mentioned trypsin solution was introduced into the air-sacs in both groups. With this doubled dose of trypsin, 80% of the controls showed necrosis of the skin above the pouch, while all the carrageenin-pretreated animals were perfectly protected. It may be concluded that an inflammatory granuloma, produced by an aqueous solution of an irritating polysaccharide, also gives protection against this enzyme.

Summary. Using the granuloma-pouch as an indicator, it has been shown that trypsin, given simultaneously with croton oil, causes particularly intense and widespread tissue-damage in the rat. On the other hand, if trypsin is introduced into the inflamed area 5 days after the croton oil, the granulomatous lining, formed under the influence of this chemical irritant, protects the adjacent tissues against otherwise toxic concentrations of this enzyme. The importance of the time-factor, in determining the effect of trypsin upon inflamed tissue, is emphasized.

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A Possible Role of Iodinated Casein in Intestinal Assimilation of Vitamin B_{12}.* (20996)

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A widely used technic for increasing responses of animals to dietary vitamin B_{12} is the dietary inclusion of iodinated casein, as outlined by Betheil *et al.*(1), and others(2,3). It apparently is generally considered that the iodinated casein is effective because of its content of thyroxine and related compounds (4,5), which by increasing the rate of metabolism of a vit. B_{12}-deficient diet, makes animals thus conditioned more sensitive to dietary vit. B_{12}. That this may not be the whole explanation is indicated by the present results.

Experimental. Fifty day-old single comb White Leghorn cockerels were placed, after one day on corn meal, on a corn-soy diet† containing no animal products. An equal number was placed on this diet to which had been added 0.05% of iodinated casein.‡ Both diets contained 1.0% of formylsulfathiazole.§ Half of the birds under each dietary regimen were subjected to a 32-hour fast at 2 days, and again at 8 days of age, in an attempt to deplete yolk-sac reserves. Thus there were 4 experimental treatments in the depletion phase of the experiment: 1) basal, 2) basal plus fasting regimen, 3) basal plus iodinated casein, and 4) basal plus iodinated casein plus fasting regimen. At age 12 days, 5 birds, and again at age 15 days 4 birds were taken from each group for measurement of the experimental criteria. Each group then contained 16 residual birds. These were divided into halves. One half from each depletion treatment was placed on the same B_{12}-deficient basal diet‖

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† The same as described previously(6).

‡ Protamone, kindly supplied by Dr. W. R. Graham, Cerophyl Laboratories, Kansas City, Mo.

§ Formo-Cibazol, manufactured by Ciba Pharmaceutical Products, Inc., provided through the courtesy of Dr. F. X. Gassner.

TABLE I. Average Body Weights (g).

Treatment	Depletion period 6/27 (Initial)	Depletion period 7/11		B_{12} comparison period 7/11	B_{12} comparison period 7/27
Basal	41 (25)	108 (20)	a	113.5(8)	285 (8)
			b	107.5(8)	282 (8)
Fasted	41 (25)	102 (21)	a	107 (8)	265.5(8)
			b	99.4(8)	254 (7)
Protamone	41 (25)	116 (20)	a	105 (8)	257.5(8)
			b	127 (8)	295.5(8)
Fasted with Protamone	41 (25)	96.2(19)	a	92.4(8)	253 (8)
			b	104 (7)	287 (7)

The "a" subgroups were continued after 7/11 on basal diet. The "b" subgroups were given this diet plus 25 μg B_{12}/kg. All differences in weight between corresponding "a" and "b" subgroups are due to selection that date, and should be kept in mind in studying weights of 7/27. Numbers in parentheses tell the number of bird weights averaged.

as used for depletion, the other half on this diet to which had been added 25 μg of crystalline vit. B_{12}¶ per kg of diet. This afforded a chance to observe response to vit. B_{12} by chicks from all 4 depletion regimens. After 2 weeks on the B_{12} response trial (at age 4 weeks) all birds were sacrificed for termination samples. Criteria determined on the termination samples were 1) body weight; 2) liver weight; 3) vit. B_{12} content of a) liver, b) small intestine plus contents,** and c) ceca plus contents; 4) and 5) coliform counts and yeast-mold counts on a) small intestine plus contents,** and b) ceca plus contents. In addition body weight, liver weight and liver vit. B_{12} were determined for the depletion samples, as well as microbial counts on excreta at 18 days of age.

Results. The results are shown in Tables I-III. It is apparent from Table I that significant growth responses to vit. B_{12} were not obtained. Liver weights were observed, but were not markedly affected by any of the treatments, except as would be expected as a result of fasting. Yet in Table II large differences in liver vit. B_{12} appear. The inference must be that the levels of liver vit. B_{12} found in the non-B_{12} chicks corresponded to adequate or more than adequate nutrition with respect to vit. B_{12}. This is in keeping with earlier experience(6). Furthermore the great increase in liver B_{12} potency against time, even in the non-B_{12} chicks, indicates that biosynthesis of vit. B_{12} was taking place in all groups.

The supposed biosynthesis might well be questioned, since in this experiment all diets contained 1% of formylsulfathiazole, which was recommended as an intestinal microbial deterrent. It is clear from Table III, however, that the ceca from birds in all treatments were well supplied with coliform, yeast, and mold organisms. (Similar counts on small intestine gave uniformly negative results.) Furthermore (Table II) the vit. B_{12} contents of the ceca were much higher than those of the small intestines. From these results it can scarcely be doubted that intestinal biosynthesis of vit. B_{12} was taking place; although the development of liver concentration of vit. B_{12} against time (Table II) supports the supposition that the sulfa compound may at first have been quite effective in reducing biosynthesis, but later lost its effectiveness, probably by the development of resistant strains among the microflora.

It would appear (Termination, Table II) that Protamone increased responsiveness of chicks to vit. B_{12} in terms of liver content of the vitamin. This effect may have been partly

‖ Birds depleted in the presence of Protamone continued to receive it. Birds depleted in the absence of Protamone did not now receive it.

¶ Through the courtesy of Dr. L. Michaud, Merck and Co., Rahway, N. J.

** Approximately 5 inches beginning at, and running posteriorly from the pancreatic duct. These, and the ceca plus contents were prepared for plate counts and vit. B_{12} assay by preliminary maceration, in sterile Waring blendors, of dilutions made in physiological saline solution.

TABLE II. Average Vitamin B_{12} Contents of Tissues.

	Depletion				Termination				
	7/8		7/11		7/27				
Treatment*	mμg B_{12} /g liver	mμg B_{12} /liver	mμg B_{12} /g liver	mμg B_{12} /liver		mμg B_{12} /g liver	mμg B_{12} /liver	mμg B_{12} /g cecum†	mμg B_{12} /g small intestine†
Basal	6.04	21.7	9.19	38.9	a	194	1410	212	12.6
					b	437	3320	190	28.5
Fasted	4.36	12.1	14.8	51.5	a	190	1260	124	15.9
					b	433	2700	129	25.6
Protamone	8.00	27.6	11.4	59.4	a	111	756	150.5	17.1
					b	465	3310	342	24.0
Fasted with Protamone	4.06	8.28	23.75	81.9	a	122	756	121	17.9
					b	374	2603	412	24.05

* Treatment shown was prior to subdivision for B_{12} response trial. "a" subgroups were controls, "b" subgroups B_{12} supplemented.

† Including contents.

a negative one, since in the non-B_{12} birds liver vit. B_{12} was reduced by Protamone from levels found in the controls. The effects within the gastrointestinal tract are seen in Table II. Note that insofar as the small intestine is concerned, the vit. B_{12} contents reflected dietary supply. Where the ceca are concerned, however, this relationship was lost *except in chicks given Protamone.* In these the ceca plus contents contained much more vit. B_{12}, consequent to dietary inclusion, than did the controls (also given vit. B_{12}, but without Protamone). This was true whether or not the birds had previously been fasted. Since it was *not* true in the absence of Protamone, the best hypothesis to account for the results is that Protamone interferes with intestinal absorption of vit. B_{12}.††

Discussion. The fact that reduction in liver B_{12} due to Protamone occurred only in the absence of added dietary B_{12} is of interest, and of possible significance in view of other work. Allen *et al.*(7), working with young dairy animals, found reduced plasma levels of carotenoids and vit. A as a consequence of feeding iodinated casein; *but only at a low carotene intake.* The effect was not detectable in other animals on a liberal carotene intake. Robblee *et al.*(3) pointed out that the effects obtained from thyroactive materials may be greatly conditioned by marginal dietary deficiencies. The thyrotoxicosis observed by them and by others in chicks at sub-marginal levels of the factor in liver anti-pernicious anemia extract and fish solubles (vit. B_{12}) may have been due to an aggravation of the deficiency of that factor by the thyroactive agents. The present results lend further support to this explanation, and suggest mechanisms by which the aggravation may be accomplished.

While the results of this experiment are not extensive, and therefore not conclusive, they shed new light on the reasons for the effectiveness of iodinated casein in rendering animals responsive to vit. B_{12} in their diets. This effectiveness has long been recognized, but never entirely understood. From the data here recorded, it appears that we must look elsewhere than to the system of endocrine interactions to fully account for the existing state of affairs. Further work along these lines is contemplated.

Summary. Dietary iodinated casein led in

†† Another possible explanation of the results must not be overlooked. This would involve two suppositions, namely (a) that Protamone under some conditions stimulates intestinal biosynthesis of vit. B_{12} in portions of the gastro-intestinal tract from which the resulting vitamin is not assimilated, and simultaneously (b) the familiar assumption that Protamone by metabolic stimulation depletes tissue (liver) vit. B_{12}; this effect being observable only at marginal nutritional status. In the absence of precise knowledge regarding assimilation of nutrients from the lower tract in chicks, liver storage capacity for vit. B_{12}, and ease of digestion and assimilation of Protamone, it is difficult to give a clear preference to either explanation suggested.

TABLE III. Microbial Counts.

Treatment*		(7/14) Excreta Coliform, cells/g × 10^6	(7/14) Excreta Yeast-mold, cells/g × 10^4	Termination (7/27) Ceca with contents Coliform, cells/g × 10^7	Termination (7/27) Ceca with contents Yeast-mold, cells/g × 10^5
Basal	a	11.0	125	2.23	2.86
	b	4.3	.24	1.18	4.09
Fasted	a	1.3	3.7	2.21	2.95
	b	.75	1.4	.385	1.52
Protamone	a	3.6	1.0	3.50	2.12
	b	15.0	1.1	.931	4.22
Fasted with Protamone	a	1.3	.18	1.24	2.09
	b	1.7	20.0	1.40	—

* Treatment shown was prior to subdivision for B_{12} response trial. "a" subgroups were controls, "b" subgroups B_{12}-supplemented.

chicks given dietary vit. B_{12} to higher cecal contents of vit. B_{12}; and in chicks not given dietary vit. B_{12} to lower liver contents of vit. B_{12} than those found in respective controls. It is suggested that the ability of iodinated casein to increase the sensitivity of chicks to dietary vit. B_{12} is due in part to an interference with intestinal absorption of the vitamin.

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Comparison of Methods for Recovering Poliomyelitis Viruses from Human Sources.*† (20997)

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The substitution of new methods for old requires an appraisal of their comparative merits. A large number of published papers attest to the usefulness of tissue culture methods for growth and immunological identification of poliomyelitis viruses, and for serum antibody surveys in the human population (1,2). In many studies stock viruses were used after adaptation to tissue culture from monkey or rodent sources. A few studies (3-6) have explored the usefulness of tissue culture methods in recovering poliomyelitis virus in specimens obtained directly from human beings.

In casting out among several methods most amenable for use in our laboratory it was quickly evident that some comparative information was required if confidence were to be

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TABLE I. Frequency of Recovery of Poliomyelitis Viruses in Tissue Culture.

Source	No. of samples	No. P	No. N	% P	Type 1	Type 2	Type 3	CP+, no type
Feces	70	47	23	67.2	43	2	2	6
Oropharynx	34	12	22	35.3	11	1		0

CP+ = Cytopathogenic in tissue culture.
P = Positive; N = Negative.

had in results obtained. The comparative information was obtained in a study of samples of feces and oropharyngeal exudate obtained from 104 individuals. The results obtained with these specimens studied in tissue cultures and in monkeys are presented in this report.

Materials and methods. Early experiences using monkey testicular roller tubes were salutary; however, with additional experience extending to other methods, monkey kidney explants were selected for general use in studies of poliomyelitis viruses. During the past year the HeLa cell type has been added to the tissue culture group. The plan of the present study was to compare recovery rates obtained with kidney and HeLa cell type tissue cultures inoculated with fecal and oropharyngeal samples obtained from human beings. Selected specimens were studied simultaneously in testicular roller tubes and in monkeys. *Feces.* Seventy fecal samples were obtained from children and adults during an illness of (a) paralytic or (b) non-paralytic (suspect) poliomyelitis, or (c) resident of a household where one or more of the members was known or suspected to be ill of poliomyelitis. Individuals identified in the first 2 categories were usually sampled during the first 2 weeks of illness. There were exceptions related to several community studies where samples from patients and familial associates were obtained 3 to 6 weeks after onset in the index case. *Throat swabs.* Thirty-four samples of oropharyngeal exudate were obtained in 1946, and stored since that time in a dry ice cabinet. The samples were obtained from patients admitted to the Children's Hospital with clinical signs of paralytic or non-paralytic poliomyelitis. *Preparation of specimens.* Feces were triturated, using sterile precautions to make a 20% suspension in distilled water. The crude extract was spun in a centrifuge (1500 rpm/10 minutes). The supernatant fluid was divided into 2 aliquots. One was used for inoculation of monkeys; the other for inoculation of tissue cultures. The latter aliquot was spun in a PR-2 International Refrigerated Centrifuge (13,000 rpm/30 min.). The supernatant fluid was usually free of bacteria. The virus was eluted from throat swabs, using a method previously described(7). Penicillin and streptomycin were added to the eluate prior to inoculation into tissue culture tubes. *Tissue cultures.* Three types of cell explants were used: (a) monkey testis, (b) monkey kidney, and (c) squamous cell carcinoma cells (HeLa) derived from a human uterine cervix. Methods used in initiating and maintaining growth of these tissues in artificial cultures have been described(8-10). Roller tubes were employed with monkey tissues. HeLa cells were grown in stationary tubes. After satisfactory outgrowth of cells each of 4 cultures of the same tissue were inoculated with 0.1 cc of the specimen under test for poliomyelitis virus. Inoculated tubes were incubated at 37°C. Tubes were read for cytolytic changes every other day. Monkey kidney and HeLa tubes were kept until the 6th day, monkey testis tubes until the 12th day, when the tests were regarded as finished. If cytolysis occurred, serial pasage was made 2, 3, or 4 times in order to exclude non-specific degenerative changes(4). The reproducibility of cytolysis having been ascertained, neutralization tests were done in order to define the strain type, using standard prototype serums prepared against 3 known immunologic types (11). *Monkeys.* Thirty-three stool samples were tested in monkeys. Twenty-four samples were tested each in 2 monkeys; 9 samples were tested each in one monkey. Stool extracts were inoculated intraperitoneally(12), and intranasally(13) in rhesus or cynomolgus monkeys. The presence or absence of poliomyelitis in monkeys was based on the ap-

TABLE II. Recovery of Poliomyelitis Viruses in 3 Different Types of Tissue Culture.

Source	Type of cells used: Monkey testicular — No. tested	No. P	% P	Monkey kidney — No. tested	No. P	% P	HeLa — No. tested	No. P	% P
Feces	35	18	51.4	70	43	61.4	63	39	61.9
Oropharynx	11	1	9.1	33	11	33.3	31	3	9.9

P = Positive.

pearance of paralysis, and the presence or absence of histologic lesions characteristically found in the central nervous system of the experimental animal.

Results. The over-all recovery rates of poliomyelitis virus from feces and orpharynx appear in Table I. Fifty-nine strains were recovered, 47 from fecal and 12 from oropharyngeal samples. The frequency distribution of types of poliomyelitis viruses are given also in Table I. Six fecal samples were cytolytic on continuous passage in HeLa cell type. Cytolysis was not abolished by specific poliomyelitis antiserum. The latter 6 samples have not been included among the positive tests.

Comparative merit of 3 types of tissue cultures. Data in regard to the relative and comparative sensitivity of each of the 3 tissue culture types appear in Table II. A significant difference was not observed in the capacity of the 3 respective tissue cultures to detect poliomyelitis virus in feces. Differences were observed in recovery of virus from throat swabs. Monkey kidney epithelium cultures provided virus recoveries 3 times as often as either testicular or HeLa cell cultures.

Among 104 samples tested 103 were tested in kidney, 94 were tested in HeLa, and 46 were tested in testicular tissue cultures. Virus was recovered from 55 samples (53.4%) in kidney, from 44 samples (45.7%) in HeLa, and from 19 samples (41.3%) in testicular cultures. In 4 instances virus was detected with either testicular (1x) or HeLa (3x) cell types and not with kidney cultures. In 7 instances virus was detected with kidney, but not with testicular (1x) or HeLa (6x) cell types.

Recovery rates and clinical category. The over-all rates of recovery of poliomyelitis virus in feces and oropharyngeal samples obtained from human beings with apparent or inapparent infection with poliomyelitis virus appear in Table III. The comparative merits of the 3 types of tissue cultures in the recovery of virus according to clinical category appear in Table IV. The over-all recovery rates signify a satisfactory indicator system(s). The high recovery rates of virus with feces obtained from patients with non-paralytic illnesses, and from family contacts support the adequacy of tissue culture methods in detecting poliomyelitis viruses. In regard to comparative recovery rates appreciable differences were not observed in fecal recovery rates obtained with any of the tissue cultures used. The observed low rates with testicular cultures cannot be considered significant because of the small number of samples. In regard to recovery of virus from the oropharynx, the rates obtained with kidney epithelium exceeded those obtained with either

TABLE III. Results in Respect to Clinical Category.

Source	No. of samples	Paralytic — No. tested	No. P	% P	Non-paralytic — No. tested	No. P	% P	Familial contacts (asymptomatic or minor illness) — No. tested	No. P	% P	Unclassified† — No. tested	No. P	% P
Feces	70	33	28	85	11	6	55	24	11	46	2	2	100
Oropharynx	34	23	10	43	6	1	17				5	1	20

* With minor illness or no history of illness.
† Hospital records unavailable.
P = Positive.

TABLE IV. Percentile Distribution of Positive Tests in Respect to Type of Tissue Culture.

Clinical category	Fecal extracts						Oropharyngeal exudate					
	Testis		Kidney		HeLa		Testis		Kidney		HeLa	
	No. P	% P	No. P	% P	No. P	% P	No. P	% P	No. P	% P	No. P	% P
Paralytic	11/16	69	25/31	81	24/28	86	1/7	14	10/22	45	4/21	19
Non-paralytic	3/ 8	37	6/11	54	4/ 8	50	0/3		1/ 6	17	1/ 5	20
Familial associates	4/11	36	10/24	41	8/23	35						
Unclassified*			2/ 4	50	3/ 4	75	0/1		1/ 5	20	0/ 5	

* Paralytic or non-paralytic patients.
11/16 = of 16 samples 11 were positive for poliomyelitis.
P = Positive.

HeLa or testicular cultures by a ratio of 3:1.

Recovery rates in tissue cultures and in monkeys. Thirty-three fecal samples were tested in monkeys. Of these samples 22 were positive and 11 were negative in tissue culture. Among the 22 positive tissue culture samples 13 were positive and 9 were negative in monkey tests. Among 11 specimens which were negative in tissue culture, 2 contained virus capable of producing experimental poliomyelitis in monkeys.

Discussion. Any one of the 3 tissue culture methods used was satisfactory for the detection of poliomyelitis virus in fecal samples obtained from patients experiencing clinical poliomyelitis. An 85% recovery rate from feces and a 43% recovery rate from oropharyngeal samplings signify an adequate indicator system in comparison with previous observations on corresponding studies in monkeys(14,15). However, there were some observed differences in the capacity of the tissue cultures to reveal virus under the conditions employed in the study. The data suggested that monkey kidney epithelium cultures were more sensitive in revealing poliomyelitis virus. To us the differences appear to be related to inocula containing comparatively fewer infective particles of virus (*i.e.*, throat swabs compared with feces) some of which may have been bound with antibody(16); or, as was pointed out by Syverton(17) another factor, difficult to properly evaluate in 2 tissue culture types because implants were used, is the comparative number of cells per unit volume available to the virus particles.

TABLE V. Comparative Tests on the Relative Sensitivity of Monkeys and Tissue Culture.

Clinical category	Monkey		Tissue culture P		Tissue culture N	
	P	N	P	N	P	N
Paralytic	7	4	1	1		
Non-paralytic	—	3	—	3		
Familial associates	6	2	1	5		
Total	13	9	2	9		

P = Positive; N = Negative.

In regard to the comparative merits of monkeys and tissue cultures in the detection of poliomyelitis virus it is evident that each method has limitations. Samples were encountered containing strains of poliomyelitis viruses which grew in tissue culture without causing cytolysis, and which produced paralytic poliomyelitis in monkeys. Conversely, there were specimens containing virus demonstrable in tissue culture and not in monkeys. In this study the type of dissociation encountered most frequently fits in the latter category. Presumably, the specimens contained small amounts of virus, for in some of them, one or 2 tubes of the 4 inoculated showed cytolysis.

No claim can be made here that tissue culture methods are more sensitive than monkeys in detecting poliomyelitis virus in specimens obtained from human beings. The data are not strictly comparable insofar as the number of test trials is weighted in favor of the tissue culture methods. It is clearly demonstrated however, in view of present and past experiences(14,15) that the tissue culture methods are as good as, if not better than, monkeys in the detection of poliomyelitis virus in fecal and oropharyngeal specimens obtained from human beings.

Summary. One hundred and four fecal and oropharyngeal specimens obtained directly

from human sources were studied in 3 types of tissue cultures. The virus recovery rates among fecal samples were of the same order regardless of the type of tissue culture used. Under the conditions of the study, monkey kidney epithelial cell cultures were superior to HeLa or testicular cultures in the primary isolation of poliomyelitis virus from oropharyngeal specimens. A comparison of tests with fecal samples in monkeys and in tissue culture indicated that the latter was as good as, if not better than, monkeys in detecting poliomyelitis virus.

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Effects of Dietary Aureomycin and Sulfasuxidine on Phosphorylation in the Liver of Rats.* (20998)

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Aureomycin has been shown to be a beneficial factor in the prevention of massive dietary necrosis in the rat(1). However, Lepper *et al.*(2) have reported that a reversible fatty infiltration of the liver frequently follows the administration of aureomycin intravenously or intraperitoneally in mice. The action of antibiotics in liver disease may be due to a direct chemical effect on the liver or to an antibacterial effect on intestinal flora. The sparing effect of antibiotics on the requirements of the rat for certain B vitamins has been shown to be mainly due to the alteration of intestinal flora(3). The supplementation of sulfasuxidine in a choline-deficient diet prevents cirrhosis(4). Both aureomycin and sulfasuxidine have been shown to delay the development of renal damage due to choline deficiency(5,6). However, no analyses were made on the phospholipide content of the liver following the administration of these antibiotics.

In view of the above findings, experiments were undertaken to ascertain if phospholipide

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metabolism was involved following the administration of aureomycin or sulfasuxidine.

Materials and methods. Male albino rats (100-110 g) were divided into 2 groups, with a control for each group maintained on a low protein-low fat(7) diet containing vitamin-free casein 5 parts, dextrin 42, sucrose 42, crisco 4, cod liver oil 1, salt mixture 4, Ruffex 2, plus the following vitamin mixture in mg per kilo of ration: dextrin 9639, thiamin 8, riboflavin 8, pyridoxine 8, nicotinic acid 241, calcium pantothenate 32, inositol 32, para-aminobenzoic acid 32. The rats of Group 1 received the low protein-low fat diet with and without supplementation of aureomycin (25 mg/rat/day for 1, 2, 4 and 8 weeks). The animals of Group 2 received the low protein-low fat diet with and without the supplementation of 5% sulfasuxidine for 5 and 7 weeks. Consumption weight of food was made daily and body weight was done every 2 days. Radioactive phosphorus was administered intraperitoneally as inorganic phosphate, containing 2-4 microcuries of P^{32}, and after 6 hours the animals were killed by decapitation. The liver was removed, rapidly weighed, and analyses made for acid soluble P, phospholipide P and nucleoprotein P and radioactivity as that previously described(8).

Results. The influence of antibiotics, aureomycin and sulfasuxidine, on lipide and nucleoprotein metabolism and phosphorus and nitrogen distribution in the liver are reported in Tables I and II. To evaluate the statistical significance of the results, the *t* test of significance(9) was applied to the differences between the mean and the results from each group. The data in Table I show that aureomycin stimulates the growth of rats maintained on a low protein-low fat diet and the *t* test showed the weight to be highly significant at 4 and 8 weeks with values of 3.7 and 6.2 respectively. The probability P for chance occurrence of these differences was <0.01. Concurrent with this increase in body weight, there was a significant increase in food consumption (*t* values of 10.9 at 4 weeks and 10.45 at 8 weeks, with a P value of <0.01). This finding is in agreement with Gyorgy *et al.*(1). However, there was very little change in the total liver lipides or the synthesis of phospholipide and nucleoprotein following the supplementation of aureomycin in the diet. Young growing rats have been shown to have an increased synthesis of phospholipides(10). The stimulation of growth following the administration of aureomycin failed to reflect an increase in the synthesis of phospholipides. After 4 weeks of supplementation of aureomycin, there was a significant drop in phospholipide synthesis when expressed as per gram of fat free tissue (*t* values of 2.99 with a P <0.01). This decrease in lipide phosphorylation may be due to a direct inhibitory effect of aureomycin (11). The ratio of the relative specific activities remained the same even if aureomycin is supplemented for as long as 8 weeks in the diet.

It is of interest that the ratio of synthesis of phospholipides to nucleoproteins is constant in the liver, irrespective of dietary fat or protein content, and is not influenced by the presence or absence of fat in the liver(12). Campbell and Kosterlitz(13) have demonstrated in the rat that the ratio of liver protein nitrogen to phospholipides remains approximately constant over various levels of dietary protein. This constant rate of synthesis of liver phospholipides appears to be changed very little by dietary intake, but may be influenced by the metabolic requirements of the body(4,14,15).

The supplementation of 5% sulfasuxidine to the low protein fat diet for 5 weeks inhibited the growth of the rats and there was a decrease in food consumption. There was a decrease in fat content of the liver following the administration of sulfasuxidine for 5 to 7 weeks. If the results were expressed as per gram of fat-free tissue, there was an increase in phospholipide synthesis. The stimulation of nucleoprotein P synthesis following the administration of sulfasuxidine is to the same extent as that of the phospholipides as shown by the ratios of the relative specific activities of the phospholipide P to that of the nucleoprotein P which is unchanged.

The mechanism of this lipotropic effect of sulfasuxidine is undetermined. The administration of 1 to 2% sulfaguanidine has been used to produce hyperplasia of the thyroid

TABLE I. Effect of Aureomycin (25 mg/Rat/Day) on Phosphorylation in Liver of Rats Maintained on Low Protein-Low Fat Diet.*

Supplement in diet	No. of rats	Body wt change, g	Food consumption, g/rat/day	Whole liver — Wt, g	Whole liver — Total lipides, g	Phospholipide P, RSA‡ /g fat free tissue	Nucleoprotein P, RSA	Phospholipide P / N	RSA‡ Phospholipide P / RSA Nucleoprotein P
					1 week				
	2	+10 ± 2	12 ± 0	4.69 ± .00	.54 ± .08	.182 ± .009	.044 ± .001	.081 ± .016	.031 ± .001	2.319 ± .406
A‡	6	+10 ± 2	13 ± 0	4.69 ± .54	.57 ± .10	.191 ± .020	.047 ± .010	.102 ± .014	.035 ± .001	1.893 ± .151
					2 weeks				
	6			5.24 ± .64	.98 ± .22	.162 ± .025	.038 ± .006	.076 ± .014	.034 ± .002	2.183 ± .343
A	6			4.88 ± .58	.63 ± .28	.157 ± .027	.037 ± .008	.066 ± .012	.037 ± .003	2.409 ± .356
					4 weeks				
	16	+17 ± 8	13 ± 1	6.98 ±1.58	1.29 ± .75	.234 ± .026	.043 ± .012	.092 ± .013	.030 ± .005	2.562 ± .294
A	24	+37 ± 7†	17 ± 1†	7.70 ± .876	1.00 ± .41	.218 ± .035	.033 ± .009†	.092 ± .014	.031 ± .005	2.388 ± .279
					8 weeks				
	12	+28 ±10	13 ± 1	7.60 ±2.27	1.82 ±1.30	.170 ± .040	.030 ± .008	.068 ± .02	.036 ± .003	2.559 ± .502
A	8	+59 ±16†	16 ± 1†	7.62 ± .71	.68 ± .14	.168 ± .019	.024 ± .004	.074 ± .013	.038 ± .003	2.299 ± .215

* Figures preceded by ± sign indicate stand. dev.
† The *t* test of significance was applied to difference between means and the P probability for chance occurrence of this difference was <0.01.
‡ A = Aureomycin; RSA = Relative specific activity.

TABLE II. Effect of 5% Sulfasuxidine on Phosphorylation in Liver of Rats Maintained on Low Protein-Low Fat Diet.*

Supplement in diet	No. of rats	Body wt change, g	Food consumption, g/rat/day	Whole liver — Wt, g	Whole liver — Total lipides, g	Phospholipide P, RSA‡ /g fat free tissue	Nucleoprotein P, RSA	Phospholipide P / N	RSA‡ Phospholipide P / RSA Nucleoprotein P
					5 weeks				
	16	+13.2± 8	11 ± 1	6.93 ±1.34	1.47 ± .79	.463 ± .190	.086 ± .038	.253 ± .094	.036 ± .016	2.084 ± .928
5% S‡	15	+14.5±12†	10 ± 1†	4.81 ± .91	.57 ± .33†	.566 ± .207	.135 ± .052†	.266 ± .108	.033 ± .014	2.410 ±1.74
					7 weeks				
	9	+ 2 ±18	11 ± 1	7.42 ±1.20	2.49 ± .84	.235 ± .027	.048 ± .007	.112 ± .011	.027 ± .002	2.107 ± .126
5% S	3	−35 ±17†	7 ± 0†	4.31 ± .77†	.47 ± .18†	.361 ± .073†	.094 ± .011†	.168 ± .015†	.026 ± .003	2.143 ± .317

* Figures preceded by ± sign indicate stand. dev.
† The *t* test of significance was applied to difference between means and the P probability for chance occurrence of this difference was <0.01.
‡ S = Sulfasuxidine; RSA = Relative specific activity.

(16). Compounds such as thiouracil, which produce hyperplasia of the thyroid, prevent experimental dietary cirrhosis(17). This lipotropic effect of sulfasuxidine apparently is not a thyroid mechanism, because hypothyroid conditions inhibit phosphorylation synthesis in the liver(8). Handler and Follis(4) concluded that the mechanism of preventing cirrhosis from the supplementation of 1% sulfasuxidine in choline deficient diet was not of thyroid or lipotropic effect as evidenced by histological changes in the thyroid and liver. The present observation of a lipotropic effect of sulfasuxidine is in agreement with its antihemorrhagic properties in choline deficiency(5), because most compounds which are lipotropic delay the development of renal damage(18).

Summary. 1. The dietary effect of antibiotics, aureomycin and sulfasuxidine, on phospholipide and nucleoprotein synthesis was studied in rat livers. 2. The addition of aureomycin (25 mg/rat/day for 4 and 8 weeks) to a low protein-low fat diet caused a marked stimulation of growth and food consumption in the rat. However, there were no significant changes in the liver lipides or the synthesis of phospholipides or nucleoproteins. 3. Addition of 5% sulfasuxidine to a low protein-low fat diet produced a significant lipotropic effect. There was a significant decrease in total lipides and an increase in lipide phosphorylation.

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Effect of Glucose Diuresis on Renal Excretion of Bicarbonate.*† (20999)

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Glucose diuresis in the normal subject greatly augments the urinary excretion of sodium (1-3). Under conditions where the main urinary anion is chloride, the loss of sodium is "covered" chiefly by a corresponding loss of chloride. The fact that there is no significant change in the excretion of bicarbonate (in spite of the fact that this anion makes up about one-sixth of the anions of the glomerular filtrate) might be explained, within the present framework of hypotheses concerning renal function, in several ways: 1) The proximal tubular diuresis does not affect the proximal tubular reabsorption of bicarbonate (*i.e.*, there is no "velocity effect"(4,5) for bicarbonate)—perhaps because of rigid control of this reabsorption by internal regulatory factors such as the serum pCO_2(6). 2) The proximal tubular diuresis increases the delivery of bicarbonate to the distal tubules, but not sufficiently to exceed a proposed distal Tm for bicarbonate(7).

In the present experiments, we studied the effect of glucose diuresis on the excretion of bicarbonate under conditions where bicarbonate made up a large proportion of the urinary anion excretion, and where, therefore, any hypothetical distal Tm for bicarbonate might well have been exceeded.

Procedure. The subjects of this experiment were 3 normal male medical students. Fluids and food were withheld from 10 o'clock on the previous evening. Each study began at approximately 8 A.M. and continued for the next 4½ to 5½ hours. The subjects remained in the recumbent position, except during the collection of urine, which was voided spontaneously in the erect posture. An infusion of 1.4% $NaHCO_3$ was administered at the rate of 1500 cc during the first 1½ hours, followed by 300 cc/hr thereafter. When the excretion of water and chloride had stabilized, (about 2 hr) 750-1000 cc of a 25% solution of glucose in water were infused, in about 30-40 min. Glomerular filtration rates were estimated from the clearance of inulin administered as a constant infusion in one experiment (R. P.), and from the clearance of endogenous creatinine in the others (J. M. and R. K.). Venous blood was drawn anaerobically with minimal stasis at intervals indicated in Table I. Urine was collected by voiding in the erect posture into a specially constructed cup. Urine from the bottom of the cup was collected over mercury, whereas the remainder overflowed into a graduate. In this way exposure to air of the urine collected over mercury was minimized. In 2 experiments, analyses for CO_2 content and pH of urine specimens collected by this method, agreed within 7% with analyses of urine specimens of the same voidings collected directly under oil. *Chemical methods and calculations.* Chemical methods were standard(8,9), or previously defined(10), except that the CO_2 content of the urine was directly determined on urine by the same method used for serum. The pH of serum and urine was determined with a Beckman pH-meter. The pCO_2 of the serum and urine was calculated from the determined values for total CO_2 content and pH, using the Henderson-Hasselbach equation, and the values of 6.1 for pKa′ and of 0.51 for α, for both serum and urine.

Results. Table I summarizes the detailed experimental data of each experiment. Fig. 1 presents the urinary electrolyte patterns of

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TABLE I. Experimental Data. Glucose diuresis during sodium bicarbonate infusions.

	Period*	Time, min.	Excretion rates, urine: H_2O, cc/min.	Na, μeq./min.	Cl, μeq./min.	K, μeq./min.	HCO_3, μeq./min.	pCO_2, mmHg	Glucose, μm/min.	Concentrations in serum†: Na, meq./l	Cl, meq./l	K, meq./l	CO_2, meq./l	Glucose, mm/l	pCO_2, mmHg	GFR,‡ cc/min.
(1) J.M.	A	68	7.9	713	212	221	670	96		(141)	(103)	(4.1)	(27.2)		(48)	186
91.8 K	B	30	4.4	504	97	178	537	103								194
	C**	35	4.9	477	122	131	382	111	558	142	97.1	4.1	33.7	4.8	47	214
	D	35	11.1	600	268	71	302	77	2020	140	93.4	4.1	32.8	23.9	47	258
	E	35	6.2	628	214	50	350	85	818	141	97.3	3.4	33.2	7.7	48	220
	F	41	3.7	582	84	103	503	95	115	143	93.7	3.8	35.1	1.4	51	195
(2) R.P.	A	41	3.8	982	282	177	760	86		(144)	(105)	(3.8)	(27.6)		(41)	167
73 K	B	35	4.0	1052	306	155	770	107								
	C**	28	16.9	1790	895	135	728	98	2930	144	101	3.5	33.9	4.7	42	203
	D	18	21.0	1615	1112	63	483	54	4710	130	86.6	3.1	32.3	56.1	46	175
	E	14	17.6	1565	915	70	440	50	3540	137	94.6	3.4	33.0	28.7	44	
	F	42	8.3	1270	514	90	730	89	1230	139	97.4	3.4	33.8	12.5	42	223
(3) R.K.	A	90	3.5	743	221	22	665	147		(147)	(107)	(4.2)	(28.2)		(48)	153
93.6 K	B	43	3.2	745	181	19	695	137								168
	C**	33	13.2	905	526	127	420	52	3170	148	103	3.7	33.7	4.5	47	207
	D	37	19.6	1190	827	61	350	73	4350	130	91.6	3.9	31.3	59.6	51	224
	E	24	8.3	832	414	55	391	88	1770	141	97.2	3.7	32.1	28.2	52	186
	F	26	4.0	640	203	82	436	108	548	146	100	3.7	34.5	10.2	52	162

* Each period is composed of 1-2 consecutive sub-periods corresponding to individual periods of urine collection. Period A followed 2-4 periods during which urine flow and rate of chloride excretion were being stabilized under influence of maintenance infusion of sodium bicarbonate (see Procedure). Glucose infusions were administered during periods designated by (**). 1000 cc of 25% sol. were given to R.P. and R.K.; 750 cc of 25% sol. to J.M.

† Serum values at *beginning* of corresponding urinary periods, except that *underlined values* were at *end* of corresponding urinary periods, and *values in parentheses* were before infusions of sodium bicarbonate.

‡ Values of glomerular filtration are based on clearance of endogenous creatinine in subjects J.M. and R.K., and of inulin in subject R.P. Simultaneous values for inulin clearance in periods C and D of exp. No. 3 (subject R.K.) were 193 cc/min. and 232 cc/min. respectively.

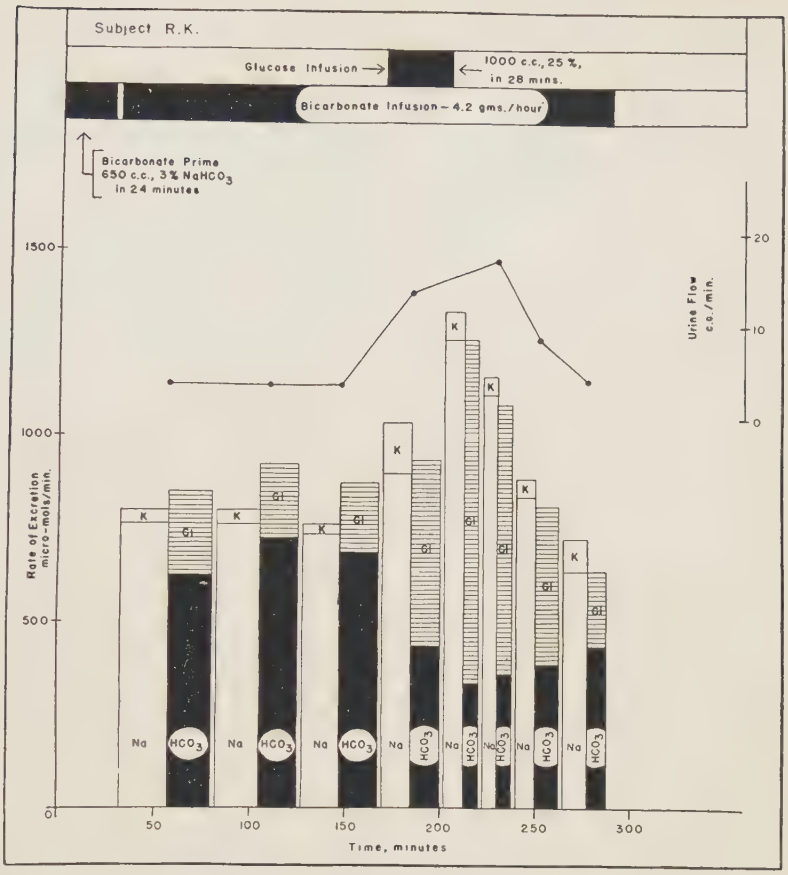

FIG. 1. Urine flows and rates of excretion of individual ions in consecutive periods of urine collection. Rate of excretion of potassium is *added* to that of sodium, rate of excretion of chloride, to that of bicarbonate.

consecutive periods of one representative experiment.

Changes in serum electrolytes. The infusions of sodium bicarbonate increased the serum bicarbonate, decreased the serum chloride, but did not change significantly the serum sodium or potassium. In the 2 experiments in which 250 g of glucose was infused (R.P. and R.K.) the serum sodium and chloride decreased as a result of the cellular dehydration caused by hypertonic glucose(3). The serum bicarbonate and pH also fell and the serum pCO_2 increased slightly. Changes in these serum values were not clearly significant in subject J. M. in whom only 188 g of glucose was infused. The serum potassium was essentially unchanged in all experiments.

Changes of urine flow and of excretion of sodium, chloride and bicarbonate during and following glucose infusion are indicated in Fig. 1 for experiment R. K. They were closely similar in the 2 other experiments (Table I). In all experiments, the excretion of chloride was increased out of proportion to that of sodium. Changes of urinary pCO_2 followed those of total CO_2 content (or bicarbonate). The excretion of potassium decreased with glycosuria in 2 experiments (in which it was initially high) and increased in the third (in which it was initially low).

Glomerular filtration was transiently increased by the glucose infusions in the two experiments in which it was measured. As a result, the filtered bicarbonate was either unchanged (subject R. P.), or increased (R. K.) at the time when the excretion of bicarbonate declined.

Discussion. In the present experiments, the

excretion of bicarbonate decreased during osmotic (glucose) diuresis, even though, in 2 cases, the filtered bicarbonate was increased or unchanged, and although the increased excretion of water, sodium and chloride in all cases indicated the probable occurrence of proximal tubular diuresis(1-3). The results do not indicate whether proximal tubular diuresis may have decreased the proximal reabsorption of bicarbonate. If this occurred, however, it was superseded by other factors tending to increase the net tubular reabsorption of bicarbonate, since the urinary bicarbonate was diminished, while the filtered bicarbonate was increased or unchanged. One such factor may have been the slight (barely significant) increase in the serum pCO_2 which occurred in the two most definitive experiments (R. P. and R. K.). Recent workers have offered convincing evidence of a direct relationship between bicarbonate reabsorption and the serum pCO_2(6).

Summary. Under conditions favoring the excretion of bicarbonate, the rate of excretion of bicarbonate decreased, while the rates of excretion of other electrolytes increased, during an osmotic (glucose) diuresis. Possible explanations for this observation are discussed in relation to present concepts of osmotic diuresis and of the excretion of bicarbonate.

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Prevention of Innominate Bone Separation During Pregnancy in the Mouse.* (21000)

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Gardner and Van Heuverswyn(1) injected large amounts of testosterone propionate into pregnant mice and found that it inhibited pregnancy pelvic changes such as the formation of an interpubic ligament and increased flexibility of the sacroiliac joints. Since the interpubic ligament did not form, the separation of the innominate bones at the symphysis failed to occur. This prevented the marked increase in size of the birth canal which normally occurs during pregnancy. Two mice died with macerated fetuses in the cervix and upper vagina, 4 gave birth to macerated fetuses, and 4 gave birth to living young. These results indicate that in some mice one or more of the pregnancy pelvic changes are necessary for normal parturition to occur. In a previous experiment(2) the interpubic tissue in Brown-belt strain, virgin mice consisted chiefly of cartilage. During the first pregnancy this cartilage was replaced by an interpubic ligament. The latter produced a 4.3 mm average separation of the innominate bones at the symphysis. The present experiment was performed to determine the necessity of innominate bone separation at the symphysis during pregnancy.

Materials and methods. Twenty virgin mice of the Brown-belt strain were used. At puberty their innominate bones were tied together at the pubic symphysis in order to prevent their separation during pregnancy.

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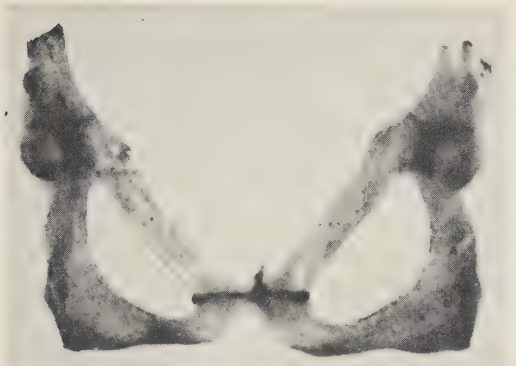

FIG. 1. A whole mount of the innominate bones and symphysis, excluding most of the ilia, from a female sacrificed immediately after her first parturition. Nine normal young were born on the 20th day. The black thread which bound the innominates together at the symphysis during pregnancy is still present. × 6.

This was done using a No. 4 half-circle eye needle and No. 000 black surgical silk thread. After exposing the symphysial area the needle was inserted through the medial portion of the obturator foramen on one side and then passed posterior to the symphysis and through the medial portion of the obturator foramen on the opposite side. The 2 free ends of the thread were then drawn tightly together and a double knot was made anterior to the symphysis. Following this, the incised skin over the symphysial area was closed by interrupted sutures. One week after the operation the females were mated. The time of fertilization was considered to be at midnight prior to the appearance of a vaginal mucous plug. Immediately following parturition the females were sacrificed. The symphysial tissue was studied grossly and histologically.

Results. Five mice failed to maintain their pregnancy beyond the early stages; 15 gave birth to normal living young. The duration of pregnancy in these latter animals was between 19 and 21 days: the usual length of time in the strain of mice used. The average number of newborn was 5: the range being 3 to 9. At autopsy the knotted thread was intact in each animal. Although the innominate bones at the symphysis were closely approximated, the symphysis was capable of some dorsal and ventral displacement (Fig. 1). The increased flexibility of the sacroiliac joints had occurred. Grossly the symphysial tissue appeared swollen. Histological examination of the symphysis revealed that the prepregnancy symphysial cartilage had been replaced by ligamentous tissue. The latter was edematous and the collagen fibers, instead of being in parallel bundles as they are in the normal symphysial ligament, were greatly disarranged.

Discussion. The results of the present experiment show that when innominate bone separation during pregnancy is prevented, the birth canal is still adequate for normal parturition as long as the pelvic joints, including the symphysis, are flexible. Since there is little strain difference in mice regarding pregnancy pelvic changes, it is probable that the present experiment could be duplicated using any strain.

The prevention of normal parturition in mice by injecting testosterone propionate during pregnancy(1) was probably due to the inhibition of the flexibility of the pelvic joints rather than the prevention of birth canal enlargement by inhibiting innominate bone separation.

The fact that the mouse symphysial tissue underwent the normal pregnancy changes on the histological level even though the lateral displacement of the innominate bones was prevented, is in accord with the findings of Van der Meer(3). When he injected relaxin into estrogen pre-treated, ovariectomized guinea pigs in which the lateral displacement of the innominate bones was prevented by the inhibition of pelvic muscle tension, the symphysial tissue became a short, swollen ligamentous mass.

Summary. The innominate bones in virgin mice of the Brown-belt strain were tied together at the pubic symphysis with silk thread. This was done to prevent innominate bone separation and concomitant enlargement of the birth canal during pregnancy. The females were then mated. Each pregnant mouse gave birth to normal living young at the usual time even though its birth canal was not enlarged during pregnancy by innominate bone separation at the symphysis. All of the pelvic joints became flexible, including the symphysis. Although the tied innominate bones remained closely approximated at the

symphysis the pre-pregnancy symphysial cartilage was replaced by ligamentous tissue.

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Sex Hormones as Protective Agents Against Radiation Mortality in Mice.* (21001)

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This report gives results of experiments on the protective effect of sex hormones against acute radiation mortality in dba mice. It has been known for some time(1,2) that in Swiss mice estrogens afford protection while androgens(2) do not. The dba mouse has been shown to be protected by the adrenal steroids, desoxycorticosterone acetate and cortisone(3). Moreover, this mouse strain showed a high sensitivity to head irradiation (3), which was perhaps attributable to injury of the pituitary(4). In view of the strain differences in radiation sensitivity seen in the course of these experiments, it was deemed desirable to examine the protective effect of sex hormones on dba mice. Since the hitherto reported protective action of estrogens was found only in preradiation-treated Swiss mice, the following experiments were carried out with postradiation as well as preradiation treatments.

Materials and procedures. a) *Mice.* Animals used in these experiments were all from our own inbred strain of dba mice. Males and females, 6 weeks old and weighing 20 g, were used. Purina Fox Chow and water were always available. b) *Hormones.* The two estrogenic hormones used were: diethylstilbestrol and α-estradiol benzoate. The two androgenic hormones were: testosterone propionate and depo-testosterone cyclopentylpropionate (the oil-soluble 17(beta)-cyclopentylpropionate ester of testosterone). c) *Radiation.* X-rays with a H.V.L. of 0.9 mm Cu had a dose rate of 150 r/min. at 30-cm target distance. The LD_{50} at approximately 12 days has been determined to be 500 r for whole-body radiation(1,2) of the dba mouse. This dose causes 100% mortality in about 20 days. The hormone was administered subcutaneously in 7 daily doses, with the 7th dose given immediately before radiation; in other animals it was given in 7 daily doses after irradiation, the 1st dose being given immediately after radiation. Table I gives the schedule of dose for each of the 4 hormones as well as the controls. For each hormone one control group had the radiation dose but no hormone while another control group was given hormone and no irradiation. The hormone dose was determined to be well below the limit of toxicity.

Results. Table I shows that the androgenic hormone provided no protection against the lethal effect of the 500-r x-ray dose. These hormones given before or after irradiation caused no change in the mortality rate. On the other hand, the estrogens reduced the mortality at 60 days after irradiation.

Fig. 1 shows that the mice without estrogen were all dead after 22 days. The effect of the stilbestrol treatment was to introduce a latent period before any animals died. This latent period was about 14-19 days for post-irradiation and 23-26 days for preirradiation administration of the diethylstilbestrol. The survival at 60 days is 35-40% for post- and 20-25% for pretreated mice.

The α-estradiol-benzoate-treated mice, Fig. 2, showed no latent period, except possibly

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TABLE I. Effect of Various Hormones on Mortality Rate Following Whole-Body Radiation (500 r) to DBA Mice.

Compound	Daily dose, mg	Time of administration, days*	Mortality at 60 days ♂ %	♂ No.†	♀ %	♀ No.†
Stilbestrol	.1	7 pre	80	20	75	20
"	.1	7 post	65	20	60	20
Control	.0	—	100	20	100	20
" ‡	.1	7	0	10	0	10
Estradiol benzoate	.09	7 pre	10	20	5	20
Idem	.09	7 post	30	20	15	20
Control	.00	—	100	20	100	20
" ‡	.09	7	0	10	0	10
Testosterone propionate	1.5	7 pre	100	15	100	15
Idem	1.5	7 post	100	15	100	15
Control	.0	—	100	15	100	15
" ‡	1.5	7	0	10	0	10
Depo-testosterone cyclopentylpropionate	.5	7 pre	100	15	100	15
Idem	.5	7 post	100	15	100	15
Control	.0	—	100	15	100	15
" ‡	.5	7	0	10	0	10

* *Pre* means daily dose given each of 7 days before day of irradiation. *Post* means daily dose given each of 7 days following irradiation, the 1st dose given on day of and immediately after irradiation.

† Total No. of animals employed in each experiment.

‡ No radiation.

the males treated before irradiation where the latent period may be 14 days. In all other animals deaths occurred beginning on the 3rd or 4th day. Fig. 2 shows that the survivals with estradiol dosage were significantly higher than with diethylstilbestrol.

Discussion. The foregoing data show that the acute radiation mortality due to 500-r whole-body dose is altered by the administration of estrogenic hormones either before or after the irradiation. There is a difference between the types of survival which result from the use of the two estrogens as protective agents. The synthetic compound, diethylstilbestrol, causes a delay in mortality, the delay being about 10 days longer when the hormone is given before radiation than when given after radiation.

The delay, or latent period, occurs in all animals treated with diethylstilbestrol, whereas it is seen only as a 9-day delay in males treated preradiation with estradiol. All other animals treated with estradiol showed the same immediate lethal effects of radiation as did the controls, although the ultimate survival with this estrogen was greater than with diethylstilbestrol. The data show that the natural hormone is more effective in dba mice than is the synthetic compound. This difference in estrogen protection is seen in the results of Graham and Graham(2) who worked with Swiss mice exposed to 400-r whole-body irradiation.

Fig. 1 and 2 show that estrogens afford protection when administered daily for 7 days after the radiation dose. This is in contrast with the results reported earlier by Treadwell *et al.*(1) and Graham and Graham(2) in which estrogens given simultaneously with or immediately after the radiation increased the lethal reaction of radiation. With the exception of 25 males given diethylstilbestrol in the Graham's report, the estrogens appear to be "toxic" to mice when administered immediately after the radiation exposure. The fact that the dba mice reported here were protected by estrogens given for 7 days postradiation raises the interesting question as to when the estrogen reaction changes from one of "toxicity" to one of protection in the interval after irradiation. Since protection is found in the dba mice and had not been found in the Swiss mice, the effect is being studied with possible mouse-strain differences in mind.

Testosterone propionate administered immediately after radiation showed a marked

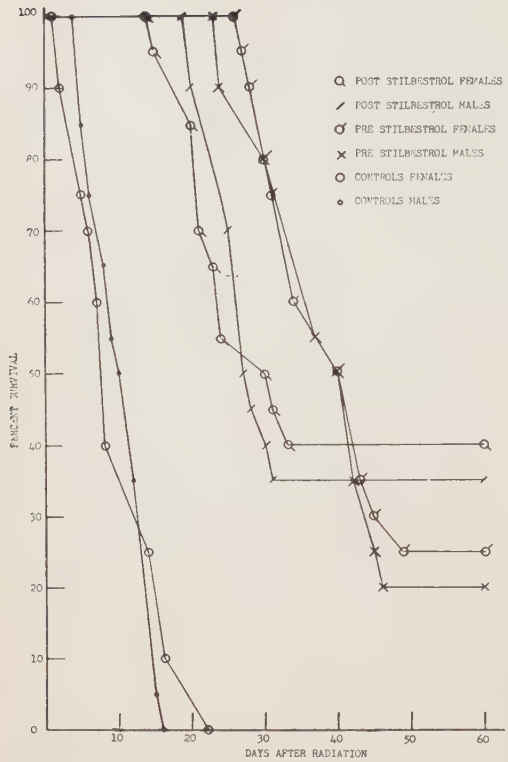

FIG. 1. Survival curves of dba mice exposed to 500-r whole-body radiation. Comparison of mice given diethyl stilbestrol for 7 days before and 7 days after radiation with controls which received no estrogen.

"toxic" effect in Swiss mice as reported by Graham and Graham(2). The same mice showed no evidence of protection when the androgen was administered 10 days before the radiation, which is corroborated by the data in Table I on dba mice. It should be pointed out, however, that the two androgens given postradiation for 7 days in our work did not increase the rate of mortality, *i.e.*, the apparent "toxic" effect reported by the Graham's was not seen in our results. However, opportunity to exhibit a toxic effect may not have been present in our experiment because of the larger radiation dose used, *i.e.*, 500 r as compared to 400 r.

With the qualification that there may be important mouse-strain differences, it appears that immediately after irradiation the mouse physiology cannot accommodate androgens and estrogens. Recovery is rapid, in the dba mouse much more rapid than the lethal processes, so that by the 7th day postradiation normal accommodation has been restored to the extent that estrogens alter the lethal processes initiated by radiation injury. Further work is in progress to determine the duration of the interval in which recovery occurs.

The postradiation administration of estrogen and its protective action on mice is of interest when compared with other substances. Cole *et al.*(5) have already pointed out that cysteine, glutathione, and sodium nitrite are effective protectors when administered before but not after irradiation. They also cite anoxia during radiation, which is protective if administered during but not after irradiation. Chemicals which are effective protectors when given postirradiation appear to be: desoxycorticosterone acetate in Swiss male mice, as reported by Ellinger(6), and in dba mice, as reported by Mirand, Reinhard, and

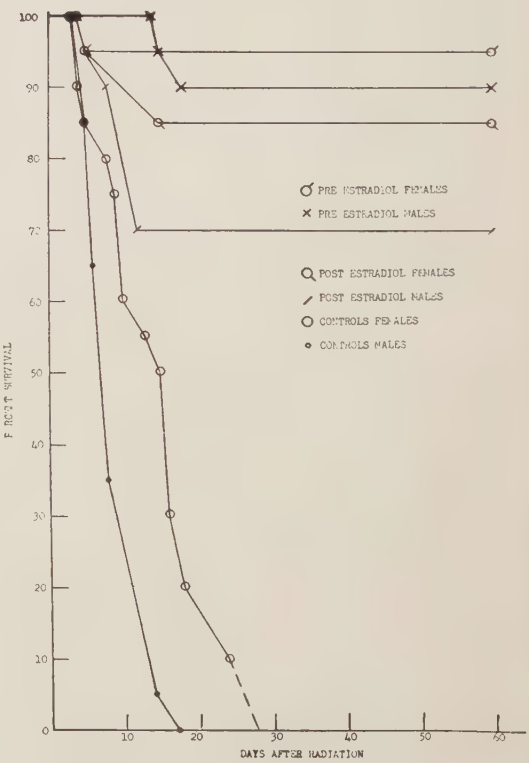

FIG. 2. Survival curves of dba mice exposed to 500-r whole-body radiation. Comparison of mice given estradiol benzoate for 7 days before and 7 days after radiation with controls which received no estrogen.

Goltz(3); the latter also reported cortisone as effective in dba mice. Jacobson *et al.*(7) found spleen transplants to be protective; this work was extended by Cole *et al.*(5) who found spleen homogenates affording protection in LAF_1 mice. Bacq *et al.*(8) mention cysteine as being a protective agent in rats if the rat liver is shielded during irradiation.

The marked ability of estrogens to ameliorate radiation injury has been noted in clinical radiotherapy. Postmenopausal patients show much more drastic symptomatic reactions than do premenopausal cases. The inference has been that endogenous levels of estrogen are sufficiently high to afford protection against radiation injury in the premenopausal state but are lacking in the postmenopause period.

Summary. 1. In dba mice diethylstilbestrol and estradiol benzoate reduce the acute radiation mortality. The androgens, testosterone propionate and depotestosterone cyclopentylpropionate, afford no protection against acute lethal radiation dose. 2. The estrogens afford protection when given for 7 days before or after the irradiation. 3. No toxic effects due to estrogens or androgens were seen in the administration of the hormones for 7 days after irradiation.

We wish to thank Dr. Kenneth Wade Thompson of Organon Inc., Dr. Robert Gaunt of Ciba Pharmaceutical Products Inc., and Dr. H. F. Hailman of the Upjohn Co. for the generous donations of hormones.

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Influence of X-Irradiation on Oxygen Poisoning in Mice.* (21002)

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The initial work on this subject already reported(1) showed a synergism between x-irradiation (8800 r) and oxygen (5-6 atmospheres) in their simultaneous effects on the survival times of mice. When intervals of 2 minutes, 30 minutes, and 2 hours were allowed between X-irradiation and oxygen poisoning, the small but significant shortening of the survival times in oxygen gradually decreased and disappeared within 5 hours. These experiments gave some support to our hypothesis on a possible common mechanism between the initial effects of X-irradiation and the effects of high oxygen tensions. When an 18-hour interval was allowed, a nonsignificant, slight reversal of this phenomenon was noticed, showing now a slight tendency to protect against O_2 poisoning. It was evident that further investigations with increasing intervals might reveal some interesting influence of the secondary phenomena of X-irradiation on oxygen poisoning.

The material presented here is a study of the effects of 8800 r (which will kill these mice in about 4 days) on the survival times of mice submitted to 6 atmospheres of oxygen

* This paper is based on work performed largely under Contract AF 18(600)-556 with the USAF School of Aviation Medicine, Randolph Field, Texas, and in part under contract with the United States Atomic Energy Commission at The University of Rochester Atomic Energy Project.

30 to 84 hours after irradiation.

Materials and methods. Chambers, made from 6-inch steel pipe which had a 3-inch window in one end and a volume of 6.2 liters, were used for testing mice. A tray of soda lime covered with a wire mesh platform with a vertical partition divided the chamber lengthwise. Transverse partitions kept the mice in the front half of the chamber, in the cone of radiation. (Two 5-volt bulbs in the chamber provided light.) In every experiment each chamber held 10 20 g female "Webster mice" of the Clara Lynch strain, 5 on each side of the central partition. A 1000 kilovolt peak General Electric Industrial X-ray Generator was used at a current of 3 milliamperes. Radiation dosages were measured with a Victoreen ionization chamber. The irradiated chamber was placed so that its center was 13½ inches from the X-ray target and the control chamber was 20 feet away, behind a lead shield. No radiation could be detected in the control chamber. Within the irradiated chamber the dose rate was 250 roentgens per minute with only slight fluctuations from day to day. The total time of exposure to irradiation was approximately 35 minutes, so that the dose of irradiation in all experiments was approximately 8780 r. The dose rate was limited by the characteristics of the X-ray apparatus. A continuous air flow was maintained in the two chambers during the irradiation. After the irradiation the mice were removed from the chambers and returned to the laboratory with food and water until they were submitted to 6 atmospheres of oxygen. The intervals in these experiments were 30, 48, 72 and 84 hours between the end of irradiation and the submission to oxygen. Each chamber held 5 irradiated mice on one side of the chamber and 5 control mice on the other side. Oxygen was supplied to the two chambers by hoses from a single cylinder of oxygen fitted with an Airco Reduction valve so that the pressure was the same in both chambers. The pressure was increased at the rate of 5 lb per minute to six atmospheres (75 psi gauge). There was a small continuous flow of oxygen through the chamber during the experiments, and the presence of soda lime kept the carbon dioxide concentration undetectable. Survival time was taken from the time 6 atmospheres of oxygen was attained until respirations ceased. In one instance the exact time of death could not be determined and the mouse was dropped from the average survival time. Observation was made of the time of onset of convulsions and exhaustion and also of the condition of the lungs at autopsy. Two experiments were done at each of the first 3 time intervals and 3 experiments were done at 84 hours, as 10 of the 30 irradiated mice had already died at that time.

Results. Fig. 1 illustrates that within five hours the synergistic effect of oxygen and radiation had disappeared, as previously reported(1). When a 30-hour interval was allowed (Table I and Fig. 1) a reverse effect was observed, showing a significant protection against oxygen poisoning by a previous irradiation. Moreover, at 84 hours when the irradiated animals had begun to die from the terminal effects of irradiation they were very markedly protected against oxygen poisoning. There was no noticeable difference in the time of onset of convulsions between

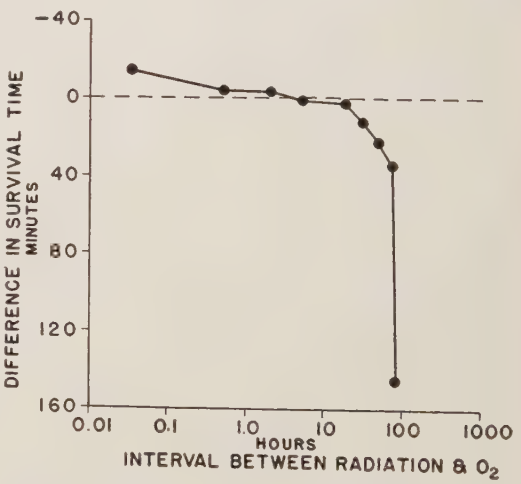

FIG. 1. Influence of initial and of secondary effects of X-irradiation on oxygen poisoning. Ordinates are differences in survival time (min.) resulting from exposure to X-irradiation compared to that from exposure to oxygen alone. Abscissae are intervals between exposure to radiation and to oxygen plotted in a logarithmic scale. Shortest interval is 2 min. Animals remained in high oxygen until death and survival times were measured from the time 6 atmospheres was attained until time of death.

TABLE I. Effect of Previous Radiation on Survival Times of Female Mice in 6 Atmospheres of Oxygen.

Interval, hr	No. of mice		% wt loss† of radiated mice	Mean survival time, min.		Increased survival due to radiation, min.	"P," %
	Control	Radiated		Control	Radiated		
30	19	20	10.0 ± 2.07	39.6 ± 1.77	51.7 ± 3.30	12.1 ± 3.75	.3
48	20	20	14.6 ± 2.09	34.2 ± 1.73	56.2 ± 3.73	22.0 ± 4.11	.0
72	20	20	22.7 ± 1.78	43.4 ± 1.73	77.4 ± 5.57	34.0 ± 5.83	.0
84	30	20*	29.3 ± 1.45	47.2 ± 1.41	193.0 ±24.29	145.8 ±24.33	.0

* Ten of 30 irradiated mice died between 72 and 84 hr, from the effects of radiation.
† Difference in final weight between control and irradiated mice in % of final control weight.

the previously irradiated and the control mice. Usually non-irradiated mice are in exhaustion approximately 20 minutes after the pressure has reached 6 atmospheres. With increasing intervals between irradiation and oxygen poisoning the time at which the irradiated mice were exhausted became progressively longer. In fact, at 84 hours some of the irradiated mice did not become exhausted until as much as 200 minutes elapsed. Only when the interval was 84 hours was a difference noticed in the appearance of the lungs of the irradiated and control mice. At that interval more than half of the lungs of the previously irradiated animals were in fairly good condition. It is interesting to mention here that Newsom and Kimeldorf(2) reported that there is an increased tolerance to lethal levels of hypoxia over that of controls following 500-600 r X-irradiation in rats. They concluded that this is, at least in part, a consequence of post irradiation anorexia as suggested by the comparable tolerance to hypoxia exhibited by non-irradiated rats fasted for 72 hours.

On the other hand, regarding increased oxygen tensions, Ozorio de Almeida reported (3) a striking increased resistance in rats starved for 4 to 6 days prior to the survival experiments in 6 atmospheres of oxygen. This finding was later confirmed by Campbell(4).

In our experiments there may have been starvation involved since the data in Table I indicate a significant progressive weight loss in the irradiated animals. Experiments are now in progress to determine to what extent the observed protection after irradiation is due to this factor.

Summary. The survival time of mice in 6 atmospheres of oxygen was about 40 minutes. The average survival times were prolonged 12, 22, 34, and 146 minutes, respectively, when the mice were irradiated (8800 r) 30, 48, 72 and 84 hours previously. Anorexia may have contributed to the observed protection.

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Physiological Mechanisms Regulating Rate of Urinary Flow in Renal Disease.* (21003)

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Normally, maximal water diuresis constitutes approximately 15% of the filtered water load, and presumably represents that fraction of the renal tubular reabsorption of water ("facultative reabsorption") which is under the control of the neurohypophysial antidiuretic hormone(1). In patients with chronic renal disease as much as 40 to 60% of the filtered water load may be excreted in the urine(2-7). Two mechanisms have been proposed to explain such excessive excretion of water in chronic renal disease: a) osmotic diuresis(2,3) and b) tubular secretion of water(4,5).

In order to help decide between these possible mechanisms, it seemed of interest to investigate several aspects of the urinary excretion of solutes in patients suffering from renal disease. Although v. Koranyi measured the freezing-point depression of urine in such patients(8), no studies of the relation between the total urinary osmotic load and the rate of urinary flow in these cases have been reported; therefore, one aim of this communication is to describe such observations made here. In addition, data on the relationships among the glomerular filtration rate, clearance ratios, and urinary concentrations of solutes are presented.

Methods. The experimental subjects were 7 patients suffering from renal disease: 4 from chronic glomerulonephritis (I.G., F.S., C.L., and S.H.), one from acute glomerulonephritis (K.R.), one from chronic pyelonephritis (P.L.), and the seventh from polycystic kidneys with severe renal insufficiency (E.S.). Four healthy, young males served as control subjects. All subjects were permitted fluids *ad lib*. The patients with renal disease received a diet containing 200 mg sodium per day, unless otherwise indicated. Twenty-four-hour collections of urine were obtained, and in addition, one-half-hour or one-hour samples of urine, obtained by catheterization, were studied in some patients. Twenty-four-hour samples of urine were collected in a bottle containing 250 mg chloromycetin and 250 mg streptomycin in order to minimize bacterial decomposition of urea. *Creatinine chromogen* in serum and urine was measured by Peters' modification of the Folin method(9), urinary and serum sodium and potassium with an internal standard flame photometer(10), chloride by the Van Slyke-Hiller modification of Sendroy's iodometric method(11), and urea by the hypobromite method(12). Measurements of freezing-point depression (Δ F.P.) of urine and serum were made by means of a Beckmann thermometer. The "effective osmolarity" was calculated as: (Δ F.P. x 1000)/1.86 = mOsm/l. The "effective urinary osmotic load" (mOsm/min.) was obtained by multiplying the urinary effective osmolarity (mOsm/l) by the rate of urinary flow (l/min.). *Reproducibility and accuracy of measurements with the Beckmann*

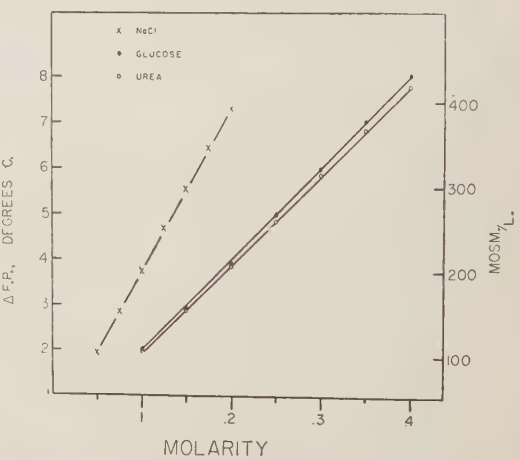

FIG. 1. Freezing-point depression of gravimetrically-standardized solutions of sodium chloride, glucose, and urea by means of the Beckmann thermometer. Each point represents mean of 10 readings on 3 independently-prepared solutions for a given concentration of each substance.

* Aided by a grant from the U. S. Public Health Service (H1245).

thermometer. With respect to the reproducibility of measurements of freezing-point depressions by means of the Beckmann thermometer, the average deviation per specimen (for 174 readings on 58 specimens) was .002°C. Fig. 1 shows the freezing-point depressions of gravimetrically-standardized solutions of sodium chloride, glucose and urea, employing 7 concentrations of each substance. Each solution was prepared independently 3 times and 10 readings were taken on the 3 solutions of each concentration. Thus, the data in Fig. 1 were obtained from 210 readings. First, it will be noted that the freezing-point depressions for sodium chloride, glucose and urea are directly proportional to their concentrations, as demonstrated by the straight lines in Fig. 1. For glucose, the slope of the line in Fig. 1 is 2.01°C/mol. which exceeds the theoretical value of 1.86°C/mol. by 8.1%. The slope of the line for urea is 1.94°C/mol. which is 4.3% greater than the calculated 1.86°C. If sodium chloride were completely ionized and there were no ionic interactions, then the anticipated freezing-point depression would be 3.72°C/mol. which is 3.2% greater than the observed slope of the line in Fig. 1, 3.6°C/mol. According to the International Critical Tables the molal freezing-point depressions for a 0.2 M solution of sodium chloride should be 3.424°C; we obtained a value of 3.665°C which is 7.0% greater. Thus, because such a high fraction of plasma osmolarity results from its content of sodium salts, our measurements of the plasma freezing-point depression are within approximately 3% of the true value. In urine where urea composes approximately one-half the total solute, the determined "effective osmolarity" in these studies is thus approximately 2% in excess of the true value. We have not made any corresponding corrections of "effective osmolarity" because they would not alter the physiological conclusions.

Results. I. *General.* Table I contains representative measurements of urinary solutes and clearance ratios in the 7 patients suffering from renal disease and in the 4 normal subjects. The plasma concentrations of electrolytes may be calculated from the data presented. It will be noted that the 24-hour

TABLE I. Representative Values for Urinary* Solutes in Patients with Renal Disease and in Normal Subjects.

Patient	C_{Cr}, ml/min.	V, ml/min.	Total solutes mOsm†/l	Total solutes mOsm /min.	Urea, mM/l	Na, mEq/l	Cl, mEq/l	K, mEq/l	Clearance ratios (%) V/C_{Cr}	C_{os}/C_{Cr}	C_{Na}/C_{Cr}	C_{Cl}/C_{Cr}	C_K/C_{Cr}	$\frac{2(Na+K)}{\text{Total solutes}}$, %	BUN, mg %	Total solutes serum, mOsm†/l
Renal disease																
I.G.	9	1.82	299	.544	—	40.0	47.6	27.3	20	17.5	5.9	9	153	45	81	345
I.G.‡	11	2.49	267	.664	—	55.8	55.0	20.0	25	17.9	8.9	12	95	57	80	337
F.S.	9	.31	389	.121	318	15.3	22.4	36.4	3.4	4	.4	.9	21	27	65	330
F.S.‡	11	.86	235	.202	67	32.9	30.5	41.0	7.8	—	1.8	2.2	68	63	38	—
C.L.	20	2.01	256	.515	—	29.2	29.9	23.0	10	8	2.0	2.8	55	38	41	324
P.L.	57	1.09	415	.452	166	50.8	43.0	32.0	1.9	2.6	.7	.9	8	36	18	310
K.R.	4	1.42	174	.247	52	23.9	21.3	27.8	36	—	6.5	7.4	174	59	83	—
S.H.	20	1.41	382	.539	—	50.5	47.7	38.0	7.1	8.1	2.5	3.2	61	46	62	333
E.S.	3	.81	291	.236	117	50.0	37.2	21.0	27	24	12	12	167	56	156	332
Normal subjects																
G.B.	97	.54	1017	.550	356	162	168	97	.6	1.8	.7	.9	14	51	22	312
H.P.	128	.48	1290	.622	472	231	207	109	.4	1.6	.6	.7	9	53	19	304
S.A.	63	.58	849	.488	213	113	113	84	.9	2.5	.7	—	—	46	13	315
R.R.	107	1.58	484	.765	—	50	69	50	1.5	2.3	.5	.9	12	41	11	—

* Twenty-four hr collections of urine. Fluids permitted *ad lib.* † $\frac{\Delta F.P. \times 1000}{1.86}$. ‡ Supplemental sodium chloride administered orally.

C_{Cr} = endogenous creatinine chromogen clearance; V = rate of urinary flow.

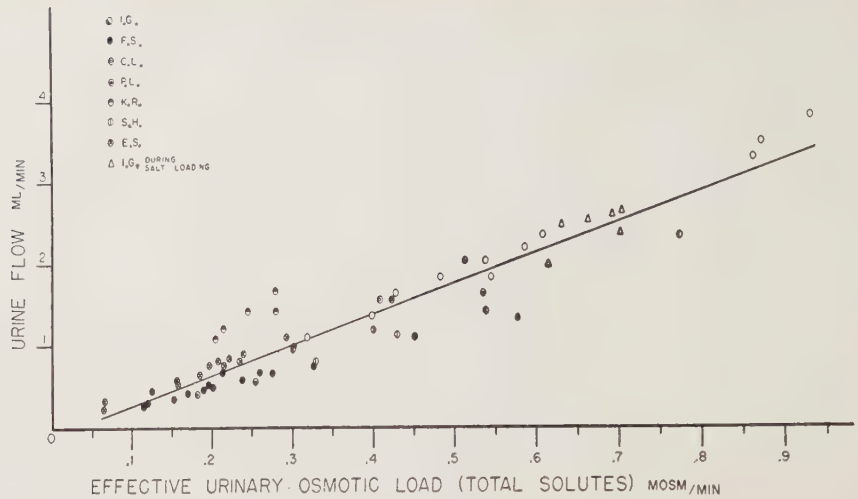

FIG. 2. Relation between rate of urine flow (ml/min.) and effective urinary osmotic load (total solutes) (mOsm/min.) in renal disease. Fluids permitted *ad lib.* K.R. is patient suffering from acute glomerulonephritis.

urinary volumes of the patients with renal disease represent much greater proportions of the daily filtered water load than those of the normal subjects (column V/C_{Cr}). The urine is hypotonic to plasma in patients I.G., F.S. (while receiving supplemental salt orally), C.L., K.R., and E.S. In all 7 patients the urinary concentrations of sodium and chloride are hypotonic to those in the plasma. It will be noted that C_{os}/C_{Cr} approximates V/C_{Cr} more closely than any other clearance ratio in the patients with renal disease. Using 2 (Na + K) mEq/l as a measure of the major portion of urinary electrolytes, the mean value for [2 (Na + K) mEq/l]/(total solutes, mOsm/l) in the patients with renal disease (47.4%) is approximately the same as the mean for the normal subjects (47.8%).

II. *Rate of urinary flow, effective urinary osmolarity, and effective urinary osmotic load.* A. *Flow versus osmotic load.* Fig. 2 shows a linear relationship between the urinary osmotic load (mOsm/min.) and the rate of urinary flow (ml/min.) in patients suffering from renal disease, including the periods during which I.G. and F.S. received supplemental salt orally. This holds for 24-hour collections of urine, as well as for one-half-hour and one-hour catheterized specimens of urine. The slope of the line, Δ y/Δ x, equals 1 ml/0.280 mOsm, indicating that the increase in urinary flow, associated with an increase in urinary osmotic load, is slightly hypotonic.

III. *Relationships among the glomerular filtration rate, clearance ratios, and urinary concentrations of solutes.* A) *C_{Cr} versus C_{os}/C_{Cr}, V/C_{Cr} and C_{Na}/C_{Cr}.* These relationships are depicted in Fig. 3. Each of these clearance ratios appears to be related to the glomerular filtration rate in a curvilinear fashion. The graph for C_{os}/C_{Cr} vs. C_{Cr} approximates that for V/C_{Cr} vs. C_{Cr} more closely than the one for C_{Na}/C_{Cr} vs. C_{Cr}. Both maximum C_{os}/C_{Cr} and maximum V/C_{Cr} exceeded 40% in this series of patients with renal disease. B) *V/GFR versus glomerular* filtration rate (GFR). Data for V/C_{Cr} vs. C_{Cr} vs. C_{Cr} obtained in the present studies are plotted in Fig. 4 along with those of Nickel *et al.*(3) who employed the clearance of inulin (C_{in}) as a measure of the glomerular filtration rate, and those of Roscoe(5) who used C_{Cr}. There is a general agreement of the results obtained in the three series of studies, in which maximum V/GFR exceeds 60% and appears to be related to the GFR in a curvilinear manner. V/GFR exceeds 15% when the GFR decreases to approximately 10 ml/min. C) *C_K/GFR versus GFR.* Fig. 5 presents the collected data on C_K/GFR vs. GFR of the present studies, as well as those of Nickel *et al.*(3) and of Roscoe(5). Again there is

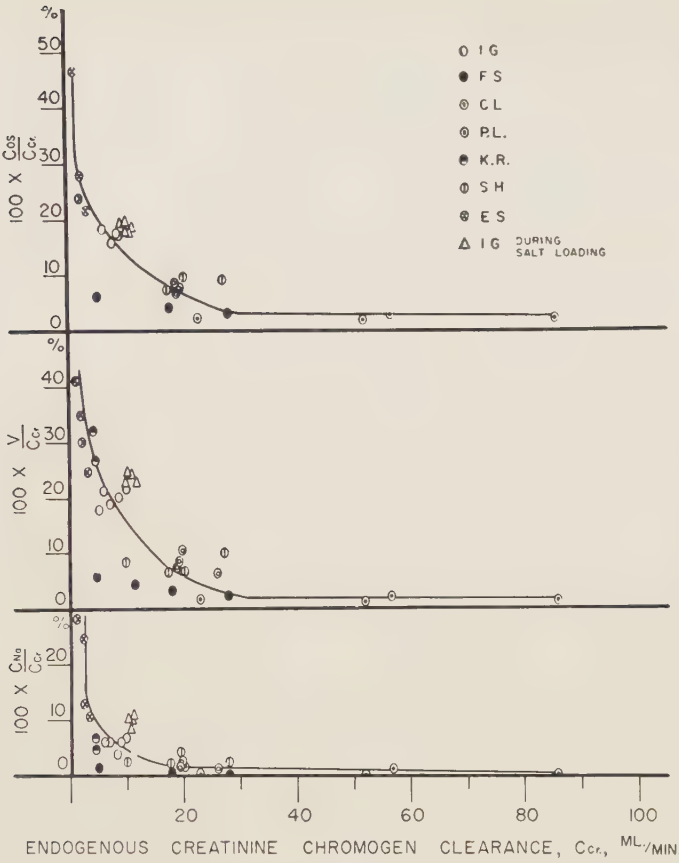

FIG. 3. Relation of clearance ratios of total solutes and sodium to glomerular filtration rate (measured by endogenous creatinine chromogen clearance). Clearance ratio of water exceeds 15% when C_{Cr} decreases to approximately 10 ml/min. or less. C_{os}/C_{Cr} vs C_{Cr} approximates V/C_{Cr} vs C_{Cr} more closely than does C_{Na}/C_{Cr} vs C_{Cr}.

a general agreement among the 3 laboratories, and C_K/GFR exceeds 100% when the GFR decreases to approximately 10 ml/min. D) *Relation of Pitressin antidiuresis to GFR.* In order to amplify the interrelationships between the glomerular filtration rate and renal function in patients suffering from renal disease, Table II presents data on the antidiuretic response of 5 other patients with chronic renal disease to a single intravenous dose of Pitressin (0.57 mU/Kg) administered during continuous intravenous hydration with 5% glucose in water(7). There is a distinct difference in the antidiuretic response between the 3 patients (S.S., W.D., Y.S.) with a GFR of 14 ml/min. or less, and the other 2 (G.F., J.W.) in whom the GFR was 19 and 24 ml/min., respectively. E) *Urinary concentrations of sodium potassium and total solutes versus* V/C_{Cr}. 1. *Sodium* Fig. 6 presents data on the relation of the urinary concentration of sodium to V/C_{Cr}. The points have also been differentiated with respect to values of C_{Cr} greater or less than 10 ml/min. There is a tendency toward a linear relationship for those instances in which C_{Cr} is less than 10 ml/min. (hollow dots), but such a relationship is not suggested for the cases with a C_{Cr} greater than 10 ml/min. (solid dots). 2. *Potassium.* Fig. 7 is a scatter diagram of the relation of the urinary concentration of potassium to V/C_{Cr}. Again the points have been differentiated with reference as to whether C_{Cr} is greater or less than 10 ml/min. A linear relationship between K (mEq/l) and V/C_{Cr} is possibly more apparent for those patients

with C_{Cr} less than 10 ml/min. (hollow dots), but is not striking in any case. 3. *Total solutes.* Fig. 8 shows the nature of the interrelationship between the effective urinary osmolarity and V/C_{Cr}, with the points also indicating whether the respective C_{Cr} is greater or less than 10 ml/min. The plateau of urinary osmolarity for the points (hollow dots) representing values of less than 10 ml/min. for C_{Cr} is apparent.

Discussion. 1. *Rate of urinary flow, effective urinary osmolarity and osmotic load.* A. *Flow versus osmotic load.* The observed linear relationship between the urinary osmotic load (mOsm/l) and the rate of urinary flow (ml/min.) (Fig. 2) suggests that osmotic diuresis is the major physiological mechanism regulating the rate of urinary flow in these patients with renal disease. The exact extent

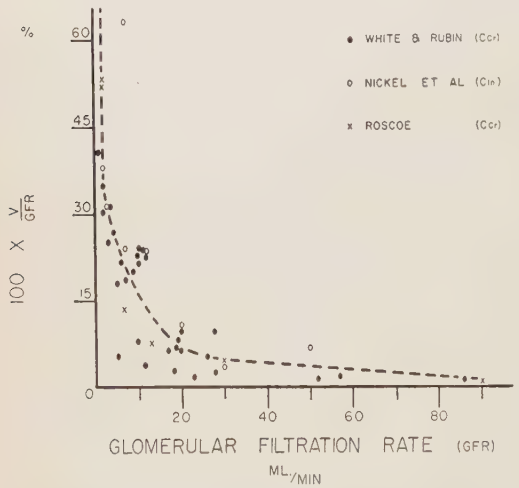

FIG. 4. Relation of clearance ratio of water to glomerular filtration rate. V = urine flow (ml/min.).

to which normal renal function must be depressed by disease for this physiological mechanism to become apparent remains to be determined. Although it has been suggested that the excessive excretion of water in patients with renal disease may be related to impaired reabsorption of electrolytes(2) or of sodium(3), reference to Table II and Fig. 3 shows a closer parallelism between V/C_{Cr} and C_{os}/C_{Cr} than between the clearance ratio of water and that of sodium or any other single solute, thus emphasizing that the excre-

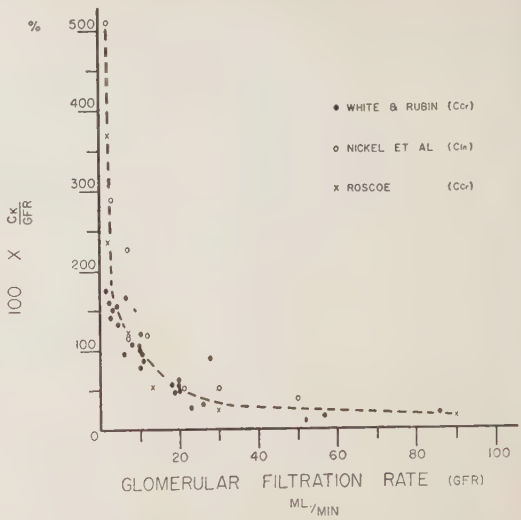

FIG. 5. Relation of clearance ratio of potassium to glomerular filtration rate.

tion of total solute is a more important determinant of the excretion of water in these patients with renal disease than is the excretion of sodium.

II. *Relationships among the glomerular filtration rate, clearance ratios, and urinary concentrations of solutes.* A. Before discussing these relationships, a comment may be in order concerning the reliability of the endogenous creatinine chromogen clearance as an estimate of GFR in patients with severe kidney disease. Table II, based on some of our earlier data(7), shows that the mean C_{Cr}/C_{in} for 5 similar patients was 1.03. Furthermore, in Fig. 4 and 5 we have plotted data taken from Nickel *et al.*(3) who employed inulin clearance to measure GFR, and

TABLE II. The Relation of Pitressin Antidiuresis to Glomerular Filtration Rate.*

Patients	GFR		Pitressin antidiuresis	
	C_{in}, ml/min.	C_{Cr}, ml/min.	% inhibition	Duration Pitressin effect, min.
S.S.	8.0	6.4	5.4	36
W.D.	13	11	6.5	30
Y.S.	14	14	0	0
G.F.	19	19	24.2	49
J.W.	24	33	31.8	46

* Data from White, Kurtz and Rubin(7).

% inhibition =

$$\frac{\text{Expected diuresis minus observed diuresis} \times 100}{\text{Expected diuresis}}$$

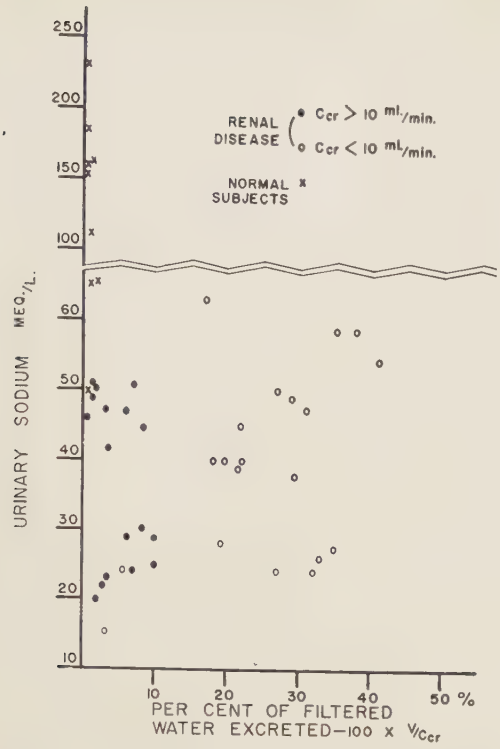

FIG. 6. Relation of urinary concentration of sodium (mEq./l) to $\frac{V \times 100}{C_{Cr}}$. A linear relationship between these variables is possibly more apparent for those patients with C_{Cr} less than 10 ml/min. (hollow dots).

it is evident that their observations are entirely consistent with ours based on C_{Cr}, as well as with Roscoe's employing C_{Cr}. It must be acknowledged that at very low glomerular filtration rates any means of estimation of the GFR may be in error. B. An interesting parallelism in the relationships between V/GFR and GFR and C_K/GFR vs. GFR, is depicted in Fig. 4 and 5. The two curves are superimposable, but whether or not this is a happenstance or represents some similarity in the handling of water and potassium by the diseased kidney must be a very speculative point with the evidence at hand. It is of interest that V/GFR exceeds 15% and C_K/GFR exceeds 100% when the GFR decreases to approximately 10 ml/min. Roscoe(5) observed that "in two normal subjects and five cases of renal failure the urinary concentrations of sodium, chloride, potassium, phosphorus, urea and protein varied with the ratio (filtration rate)/(urine volume), the relationship in each case being of the form: urine concentration = A. (filtration rate)/urine volume + B". In Fig. 6, 7 and 8 are plotted similar calculations for sodium, potassium and total solute, employing data obtained from our patients with renal disease. Inspection of these scatter diagrams does not seem to demonstrate the linear relationships described by Roscoe(5).

Summary and conclusions. 1. A linear relationship was observed between the urinary osmotic load (mOsm/min.) and the rate of urinary flow (ml/min.) in 7 patients suffering from renal disease. This suggests that osmotic diuresis is the major physiological mechanism regulating the rate of urinary flow in these patients with renal disease. 2. In these patients with renal disease there appears to be a "critical" level of the glomerular filtration

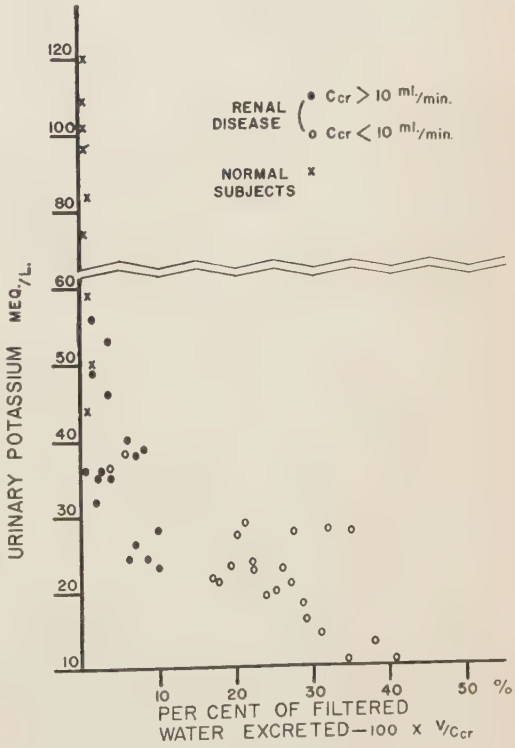

FIG. 7. Relation of urinary concentration of potassium (m.Eq./l) to $\frac{V \times 100}{C_{Cr}}$. A linear relationship between these variables is possibly more apparent for those patients with C_{Cr} less than 10 ml/min. (hollow dots).

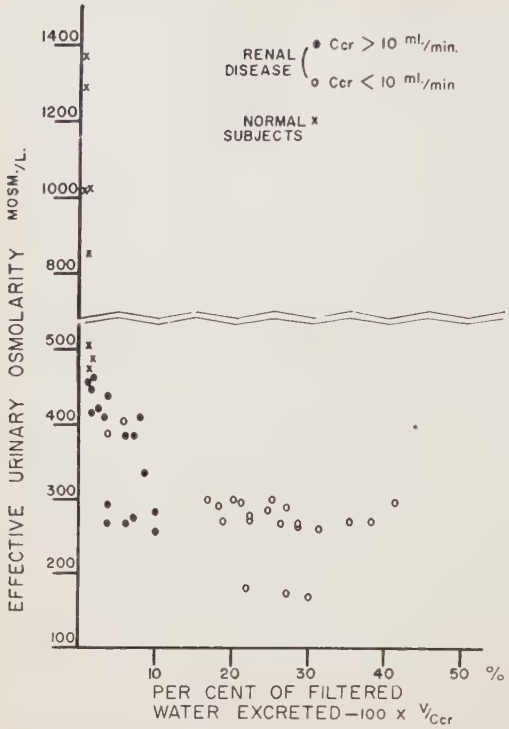

FIG. 8. Relation of effective urinary osmolarity (mOsm/l) to $\frac{V \times 100}{C_{Cr}}$. There is an apparent heterogeneity of populations, depending upon whether C_{Cr} exceeds or is less than 10 ml/min. rate, approximately 10 ml/min., at which: a) C_K/C_{Cr} exceeds 100%; b) V/C_{Cr} exceeds 15%; c) the antidiuretic response to single intravenous injections of pitressin in physiological dosage (0.57 mU/Kg) is markedly diminished; d) there is an alteration in the relationship between V/C_{Cr} and the urinary concentrations of sodium, potassium and total solute.

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Effect of Total Body Irradiation on Rabbit Pituitary as Measured by Gonadotropin Response in Chicks.* (21004)

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Certain lines of evidence have suggested that endocrine imbalances occur following total body irradiation. Botkin(1) found in the rat thyroid following irradiation an initial 24-hour upsurge of activity followed by a progressive decline until the 6th day. Response after that time was not reported. Monroe(2) reported an initial increase under the same conditions but that on the 7- and 30-day analyses the response was the same as in the controls. Both investigators used as an index of "activity" the ability of the rat thyroid to incorporate I^{131} into its protein bound fractions. Patt(3) reported a progressive increase in adrenal activity which was measured by an increase in gland weight. Early reports in the literature by Witschi(4) showed that male rats could be sterilized by exposing the scro-

* Published with permission of Director of the University of Tennessee Experiment Station.

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tum to X-rays. It was also found that the histologic response of the pituitary to X-ray sterilization was similar to that shown by castrated animals. The delicate interplay between the adrenotropin, thyrotropin and gonadotropin principles of the pituitary and the target glands is well known. It is therefore reasonable to assume that any demonstrated responses of these glands to ionizing radiations may be, in whole or in part, reflections of pituitary sensitivity to radiations.

Since a bio-assay of the pituitary gonadotropins from irradiated animals has never been reported so far as is known, it was the purpose of the present investigation to determine the effect of total body, cobalt-60 irradiation on the rabbit pituitary as measured by the chick gonad method.

Method. Female rabbits of mixed breeds, 1-2 years of age, were irradiated with Co-60 gamma rays on the multicurie irradiation site as described by Wilding(5). The radiation flux was 47 r/hr. Two groups of rabbits were exposed. One group of 40 animals received 750 r of total body irradiation and were sacrificed at 1, 8, 15 and 28 days post-irradiation. A second group of 40 rabbits was exposed to 1100 r and sacrificed at 1, 7, 12, 17 and 30 days post-irradiation. A third group of 10 normal nonirradiated rabbits was sacrificed at random during these intervals. Immediately after sacrifice the pituitaries were removed and kept frozen until all of the animals had been sacrificed. Each group of pituitaries was homogenized and diluted with distilled water so that each volume contained one pituitary per 2.5 ml of suspension. The following procedure for assay has been described in detail by Lane and Paysinger(6). One-day-old White Leghorn cockerels, obtained from a commercial hatchery were placed in an electrically heated brooder and maintained on a corn-cob meal ration during the test period.

The chicks received 5 daily injections into the axillary region with 0.25 ml of the homogenate to give a total of 1.25 ml which represented ½ of a rabbit pituitary. The birds were sacrificed on the 6th day and the testes removed and weighed. The testes weight changes were considered as the index of gonadotropin activity. It should be noted, however, that the estimate of potency was measured on a fractional gland basis rather than on a constant weight of pituitary tissue.

TABLE I. Chick Testes Weight Response to Pituitaries of Rabbits Exposed to Cobalt-60 Gamma Radiation.

No. of chicks*	Dose	Days post irradiation	Mean testes wt (mg) ± stand. error
11	750 r	1	15.70 ± 1.05
10		8	12.20 ± .48†
16		15	17.51 ± .98
16		28	18.50 ± 1.60
18	Control	—	16.20 ± .92
9	1100 r	1	13.30 ± .64‡
16		7	13.21 ± .76‡
9		12	14.30 ± .78
14		17	16.10 ± 1.18
9		30	16.30 ± .70

* Each pituitary assayed in 2 chicks.
† $P < 0.01$.
‡ $P < 0.05$.

Results. The response of the chick testes to the gonadotropin principle from the pituitaries of the normal and irradiated rabbits is shown in Table I.

The pituitaries from the 750 r treated group reflected a significantly lowered gonadotropin activity on the eighth (8th) day post irradiation (t test $P < .01$). On the other days (1, 15 and 28) no significant change from the normal level was observed.

In the 1100 r treated animals the suppression was evident as early as day one and persisted through day 7, ($P < .05$). The 12th day indicated a continued suppression but was not substantiated by statistics. The 17th and 30th day values were within the control range.

The experiment herein described presents preliminary evidence that there is a suppression of gonadotropin principle within the pituitary following total body irradiation. Although only one-tropic activity was measured, its suppression at one day following irradiation (1100 r group) is in marked contrast to the increased tropin activity of the thyroid and adrenal gland as previously mentioned. The activity of these principles within these target organs is no certain indication of the status of these stimuli within the pituitary. However, the early transgressions from the norm of these various tropins are probably

an indication of an initial stress reaction of the animal to ionizing radiations.

Storer(7) found that the testes weight of rats exposed to 500 r total body X-rays was decreased to approximately 74% of the control weights 4 weeks after irradiation. In both experimental groups reported here the gonadotropin activity of the pituitary at 4 weeks was not significantly different from the controls. It seems therefore that the testes damage observed 30 days after irradiation may be due to damage *in situ* and not the result of any continued alteration of gonadotropin activity within the pituitary. Neither can one exclusively reconcile the gonadotropin activity with histologic changes in the pituitary (*e.g.* castration cells) for in all probability the cellular changes observed are the result of a severe imbalance of several endocrine principles.

Summary. Rabbit pituitaries from animals exposed to 750 and 1100 r of total body Cobalt-60 gamma irradiation showed an early suppression of gonadotropin principles as measured by the chick gonad response. The 750 r group showed the depression on the 8th day after irradiation while in the 1100 r group the suppression was evident by day one and was sustained through day 7. After this time the gonadotropin activity in both groups returned to the normal level.

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A Hemagglutination Inhibition Test for Infectious Sinusitis of Turkeys.* (21005)

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Infectious sinusitis (IS) of turkeys is prevalent and of considerable economic importance in the United States and Canada. The cause of this disease is now generally considered to be a pleuropneumonia-like organism (PPLO) (1,2). Egg transmission of the infectious sinusitis agent has been shown to occur and this means of spread appears to play a major role in the dissemination of the disease(2-5). Efforts to devise a serological test, employing *in ovo* neutralization and cold hemagglutination, for use in the study of this disease, have not succeeded(6).

It has been shown that PPLO derived from chickens cause agglutination of chicken red blood cells and that sera of chickens infected with these organisms possess the ability of inhibiting this reaction(7,8). In this paper are presented the results of testing the sera of 4 turkey flocks for the presence of hemagglutination inhibition (HI) antibodies.

Materials and methods. The methods of isolation of the PPLO used in this study are essentially the same as outlined for the isolation of PPLO from chickens(9), except that the organisms were isolated from sinus exudate of infected turkeys. The blood samples were obtained at commercial killing plants in Ontario from turkeys which were from 26-50 weeks of age. In principle the test was performed in the same way as the HI test for Newcastle disease(10). The technic of performing this test has been described in detail

* This project is supported jointly by the Ontario Veterinary College and the Connaught Medical Research Laboratories.

TABLE I. Hemagglutination Inhibition Antibodies in Turkey Sera.

Flock	No. of turkeys ×100	Age, wk	Breed	No. of sera tested	HI titers*									% positive sera	Clinical disease†	
					<5	5	10	20	40	80	160	320	640		Sinusitis, %	Respiratory symptoms
L	30	50	WH	144	13	23	54	31	15	6	2			90	0	Marked
	180	32–35	BBB	126	2	1	5	9	46	50	7	4	2	98.4	60	”
D	18	30	Neb.	33	3	0	3	9	5	10	2	1		90.9	25	Slight
C	16	30	BBB	38	38									0	<1	None
W	21	26	BBB	73	73									0	0	”

* Expressed as reciprocals of highest serum dilution causing complete inhibition of hemagglutination. (<5 = negative.)
† Based on observations during entire growing period.
WH = White Holland, BBB = Broad Breasted Bronze, Neb. = Nebraskan.

previously(11). A single strain (isolated from flock D) was used to conduct these tests.

Results. The results of testing the sera of 414 turkeys from 4 flocks are presented in Table I. The titers reached in the positive flocks were in the range of 5-80, but sera with titers up to 640 were found.

The incidence of sinusitis in the flock is an estimate based on observations in the killing plant and in the field. Sinusitis was first noticed in flock L when the BBB birds were 8 weeks of age. Although the WH birds in the flock were hatched and raised in the same buildings as the BBB birds no sinusitis developed in this group. However, the WH poults, in common with all other birds on the premises, did develop a severe respiratory infection around 12 weeks of age. The respiratory symptoms persisted for 5-6 weeks and caused considerable unthriftiness.

The incidence of sinusitis in flock D was determined 4 weeks prior to killing, since at this time the author visited the flock and injected each infected bird with streptomycin. An occasional bird with sinusitis was seen in flock C but this developed when the birds were around 20 weeks of age and the failure to detect any positive sera may be due either to the small sample size and/or to the fact that none of the birds which were bled showed any evidence of sinusitis. No sinusitis was observed in flock W at any time during the life of the birds. Prior to the choice of strain D as the test strain, tests were performed to determine if the avian PPLO were antigenically homogeneous with respect to the HI test. A strain of PPLO isolated from flock L was studied in order to compare the sera of flocks L and D with homologous and heterologous antigens. Two other strains obtained from chickens were used to extend those observations. Tests on 15 sera from each flock indicated that if a serum were positive it would have the same titer independent of the PPLO strain used for antigen. Conversely, sera showing no HI antibodies with one strain were negative with the other 3 strains tested. In the sera obtained from flock L it was noticed that an inhibitory substance was present in 9 of the 126 BBB sera tested. This substance prevented the inhibition of agglu-

tination by positive sera at the 1/5 dilution. However, it did not interfere with the interpretation of the tests and could not be destroyed by heating at 56°C for ½ hour. No such interference was observed with the sera from flock D or with the WH sera from flock L.

The reproducibility of the test over varying periods of time was examined. Sera from flock L (BBB) were tested when strain D was at the 6th broth passage and again when the strain was at the 15th passage, or 8 weeks later. The serum titers on both occasions showed almost perfect agreement except for a few sera where the titer dropped from 160 or 320 to 80. This may be due to deterioration of antibody over the period of storage at cold room temperature. A frozen pooled sample was used as a positive control and was tested at each passage level from the 6th to 15th. The titer of this standard serum was usually 40, but occasionally 80. Such variation is inherent in the test.

Discussion. This study was initiated with the hope that a diagnostic test could be devised which would be of practical value in the selection of PPLO-free breeding stock. The results presented indicate that a hemagglutination inhibition test might serve this purpose. Although testing large numbers of sera from breeder flocks would be tedious, the advantages of obtaining disease-free replacement stock would more than repay this effort. It would appear that destroying those birds showing sinusitis does not serve to eliminate the disease from a flock since a large number of birds have been found with PPLO antibodies, but no evidence of sinusitis. Although the WH birds in flock L did not develop sinusitis, they were nevertheless affected with a chronic respiratory condition. The serological results indicate that PPLO were involved in both these conditions. The factors governing development of sinusitis in a bird infected with PPLO are obscure, but it is apparent that infection with PPLO does not necessarily result in sinusitis.

Summary. 1. Four hundred and fourteen sera from 4 turkey flocks have been tested by means of hemagglutination inhibition test utilizing a PPLO antigen. 2. This test gives reproducible results and gives promise of being useful in screening breeder flocks. 3. PPLO isolated from chickens and turkeys are apparently antigenically homogeneous with respect to this HI test as based upon 4 strains isolated from widely separated sources.

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Effect of Aminopterin on Radioactivity of Rat Liver- and Intestinal-Ribonucleic Acid after C^{14} Formate Injection.* (21006)

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During the course of experiments on the metabolism of C^{14} formate in rats the effect of aminopterin injection on the incorporation of the carbon of formate into ribonucleic acid purines was determined. The experiments were conducted somewhat differently from those of Goldthwait and Bendich(1) and an increase in the radioactivity of the liver ribonucleic acid purines was found instead of a decrease as reported by those authors. Skipper, Mitchell, and Bennett had previously made similar experiments on mice(2) and likewise had reported a reduction of formate incorporation following aminopterin injection.

Methods. For the present experiments 6 male Sprague-Dawley albino rats weighing 90-110 g each were employed. Two of these animals served as normal controls while two were injected with 100 μg of aminopterin daily for 4 days. The remaining two served as inanition controls and were pair-fed with the aminopterin treated animals. All animals received a purified diet similar to that used by Kelley *et al.*(3). *White blood cell* counts were made by standard procedures on each of the animals several times during the course of the experiment. The results of the counts are recorded in Table I.

Sixty-eight hours after the beginning of aminopterin treatment each animal received by intraperitoneal injection 10 μc (10 μ moles) of C^{14} sodium formate. Nineteen hours later all animals were sacrificed and samples of liver and small intestines taken for analysis.

TABLE I. White Blood Cell Numbers of Controls and Aminopterin-Treated Albino Rats.

Time after aminopterin treatment, hr	Controls: Food *(ad lib)*	Controls: Pair-fed	Aminopterin-treated
	Thousands/μl		
−24	12.9	14.3	15.8
	15.6	16.3	19.8
+37	12.5	23.4	10.5
	16.4	17.8	15.1
66	16.1	20.2	8.4
	19.6	16.5	9.6
87	21.3	17.8	5.9
	17.3	16.0	1.6

The tissues were homogenized in 5 volumes of water and 5 ml of the homogenate used for the analyses, which were carried out in duplicate. The fractionation procedure of Schneider(4) as modified by Ogur and Rosen (5) was employed. Following removal of the acid-soluble materials with trichloroacetic acid and the phospholipids with alcohol and alcohol-ether, the ribonucleic acid was extracted with cold 1 N $HClO_4$. Most of the $HClO_4$ was removed by precipitation with KOH and aliquots of the supernatant fluid evaporated to dryness on glass plates and counted with an end-window Geiger tube and scaler. Counts were corrected for background

TABLE II. Radioactivity of Intestinal and Liver Ribonucleic Acids (RNA) and Proteins 19 Hr after C^{14} Formate Injection into Control and Aminopterin in Treated Albino Rats.

Treatment	Specific activity: cts/min./μMol RNA P*: Liver	cts/min./μMol RNA P*: Intestine	cts/min./mg protein: Liver	cts/min./mg protein: Intestine
None, controls	10.0	413	24.5	42.8
	3.5	294	14.5	20.2
Pair-fed "	15.0	325	24.0	24.0
	13.0	426	36.5	37.0
Aminopterin inj.	231	231	38.	22.3
	205	233	32.6	21.7

* Values for nucleic acid phosphorus calculated using a molar absorption of 10000 at 260 mμ.

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and, where necessary, for self absorption.

The desoxyribonucleic acid was extracted with 1.0 N $HClO_4$ at 70°. This extract, unlike the ribonucleic acid extract, was found to be grossly contaminated with radioactivity which could not be attributed to the purines or the thymine. The residual protein was washed with water, alcohol and ether and the dried powdered material counted on glass plates. Data on the protein radioactivity are included in Table II for comparison with the RNA counts.

As shown in Table II the aminopterin treatment appeared to reduce somewhat the formate incorporation into the intestinal ribonucleic acid. These results are in accord with those of Goldthwait and Bendich(2). On the other hand the liver RNA in the aminopterin-treated animals was 12-60 times more radioactive than that of the controls. Confirmation of this result was obtained in another experiment with 4 additional animals. Furthermore, the purines from the hydrolyzed RNA were isolated by precipitation as silver salts. The regenerated purines contained substantially all of the counts originally found in the RNA extracts. That the animals were affected by the aminopterin in the expected way is indicated by the lowered white blood cell counts shown in Table I.

Two possible explanations for the results on liver RNA suggest themselves. If aminopterin interferes with the formation of endogenous formate, the liver formate "pool" may be much reduced in size after a few days of treatment. Consequently, the dilution of the added radioactive formate would be much smaller in the treated animals and the specific activity of newly-formed purines correspondingly high. In addition to this effect it is possible that the reduction of active formate to methyl groups is much more sensitive in the rat to aminopterin inhibition than is purine formation. Such an effect might well divert active formate from methyl group or serine synthesis to purine synthesis. A result similar to this has been found in rabbit bone marrow *in vitro*(6).

It appears that whole animal experiments such as these will not be satisfactory until more complete knowledge of pool sizes of all intermediates, as well as net synthesis data are available.

Summary. Treatment of albino rats four times with 100 μg of aminopterin during a 68-hour period resulted in a severely lowered white blood cell count. The treated animals showed a reduced uptake of C^{14} formate in intestinal ribonucleic acid while their liver ribonucleic acid showed a markedly increased specific activity as compared with controls.

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Relationship of Form and Frequency of Experimental Epileptic Discharges to Age. (21007)

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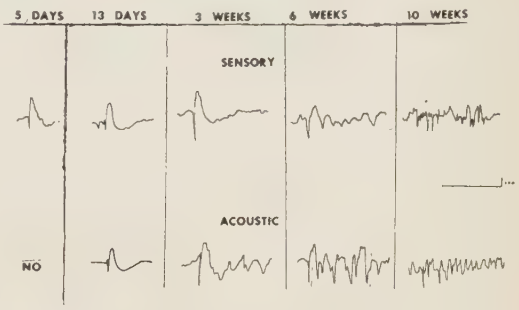
It was shown previously(1) that the development of the cortical activity in the cat proceeds postnatally according to a temporal and spatial order. The "primary" projection areas are the first to be electrically active and influenced by external stimuli, to be followed by other cortical areas. Ontogenetically, the "primordial" specific response of the cortex gradually assumes the form of a high voltage spike (followed by a single slow component under conditions of barbiturate narcosis).

The tendency of the immature cortex of the cat under barbiturate narcosis to respond to physiological sensory stimuli with a form of discharge seen in the EEG of epileptic children is of considerable experimental and clinical interest. Because the mechanism of the production of seizure discharges in general and the "spike and wave" pattern in particular is obscure, it seemed pertinent to investigate the rôle of the maturation factor in such a mechanism. Findings reported below seem to indicate convincingly that there is a definite relation between the various forms of epileptic seizure discharges and the ontogenesis of the electrical activity of the cortex.

Methods. 37 kittens distributed chronologically over the 3 postnatal months and 8 adult cats were anesthetized with nembutal or dial (2 to 5 mg per 100 g of weight, intraperitoneally). The electrocorticogram was secured through wick electrodes led into a 8-channel Grass electroencephalograph supplemented with a dual beam oscilloscope. The acoustic stimulus was a click produced by a 1 m second square wave led into an earphone with a repetition rate of .5/sec. Metrazol (Pentylenetrazol) (10%) was applied topically or was given intraperitoneally in small doses (as required to reach the desired state of excitability). "Monopolar" recording was obtained with a reference lead on the cut scalp. Surface stimulating electrodes on the cortex were bipolar with a separation of 3 mm. A Grass stimulator model S4A with all parameters variable was used for electrocortical stimulation.



EVOKED CORTICAL ACTIVITY AT VARIOUS STAGES OF POSTNATAL DEVELOPMENT

FIG. 1. Evoked responses recorded from specific somesthetic and acoustic cortex at various postnatal age. Note lack of acoustic response at 5 days.

Results. As seen in Fig. 1, at 5 days postnatally, the somatosensory cortex shows well established responses to afferent stimuli. At 13 days responses are elicited in the acoustic cortex and under moderate barbiturate narcosis both the sensory and the acoustic areas respond with a well developed "spike and wave" discharge. With increasing age the response becomes more and more complex. The "primary" spike response decreases in voltage and is followed by an "after-discharge".

It was found that the results of application of a convulsive agent like metrazol depend on the age of the preparation. In the first weeks of postnatal life of the kitten, electrical seizure activity induced in the specific areas of the cortex show characteristically repetitive "spike and wave" complexes without admixture of "fast" spike activity. At 3 weeks (Fig. 2A) the focal "spike and wave" after-discharge, induced in the left sensory cortical area by tapping the right forepaw (after topical or intraperitoneal application of metrazol), spreads slowly to the adjacent areas (acoustic and suprasylvian) and is confined to the homolateral hemisphere. At 6 weeks, (Fig.

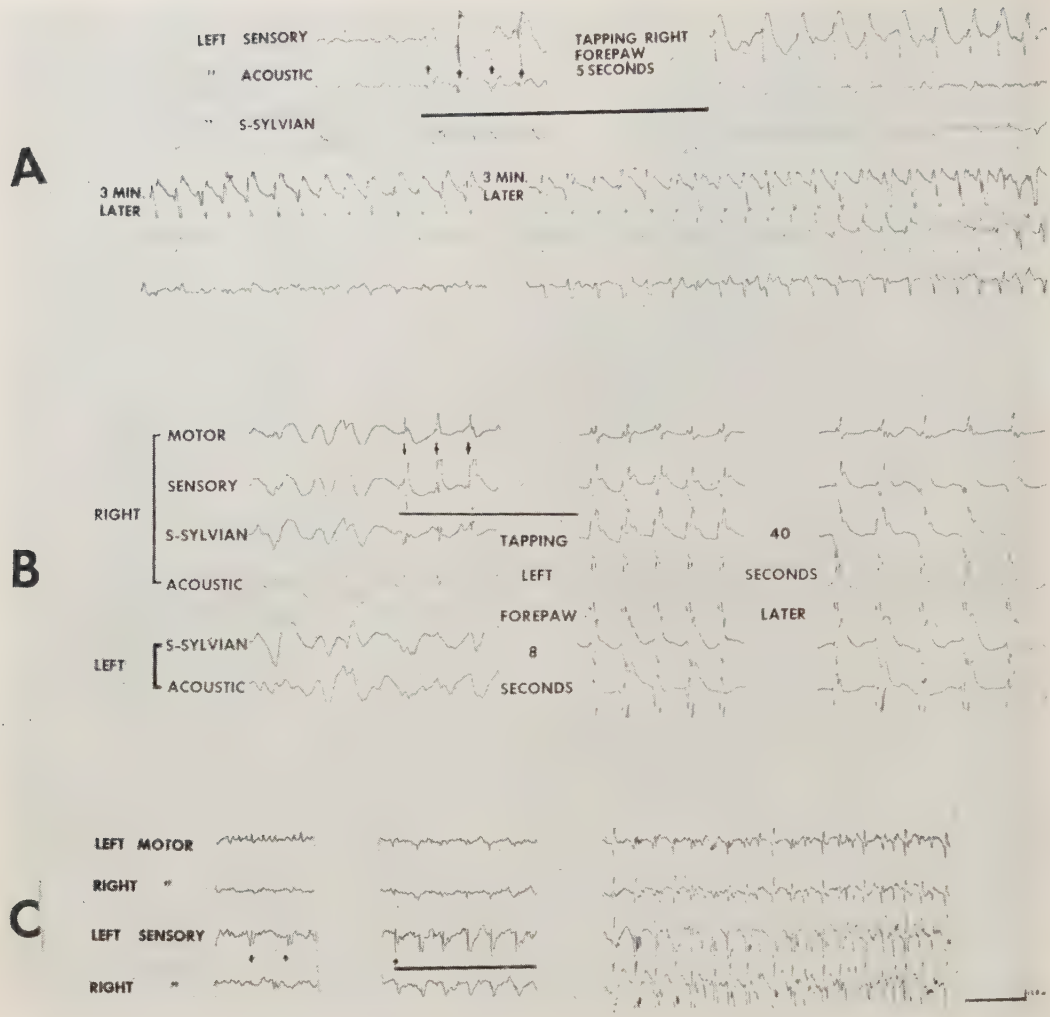


FIG. 2A. Self-sustained after-discharge in a 3-wk-old kitten evoked by stimulation of the right forepaw after topical application of 10% metrazol on left sensory cortex.

FIG. 2B. Bilateral spread of after-discharge evoked in a 6-wk-old kitten after intraperitoneal injection of metrazol and stimulation of left forepaw.

FIG. 2C. Responses to tapping of right forepaw before and after gradual increase in dose of injected metrazol. Kitten 12 wk old.

2B) after intraperitoneal application of metrazol and tapping of the left forepaw, seizure discharges are induced in the right sensory area. The seizure discharges show a rapid spread and are displayed bilaterally and synchronously in both hemispheres. At this age the "fast" spike activity is still not brought out and the seizure activity consists of "spike and wave" complexes. Only with further ontogenetical development the "fast" frequencies begin to appear more prominently during an induced generalized seizure (Fig. 2C). Such a fast activity is an almost invariable component of any generalized epileptic after-discharge produced in an adult animal.

The same tendency of the cortex to respond with a "spike and wave" discharge is revealed at earlier stages of development, by direct electrical stimulation of the cortex, without the use of a convulsive agent. In Fig. 3A typical "after-discharge" that follows the

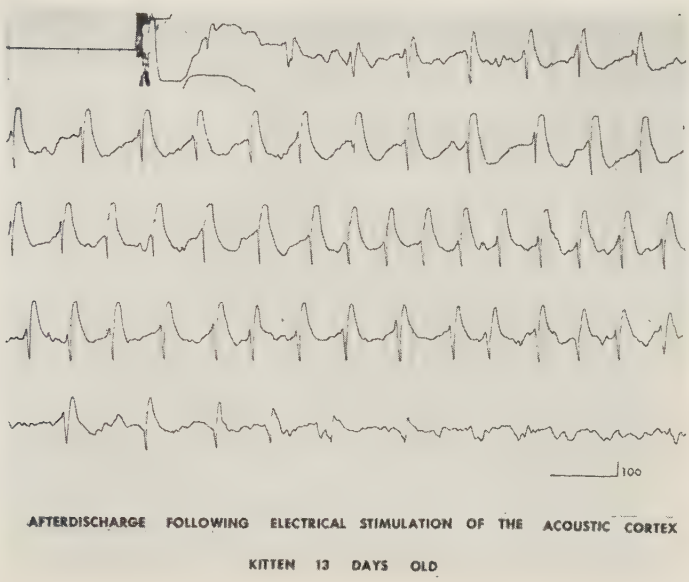


FIG. 3.

electrical stimulation of the acoustic cortex in a 13-day-old kitten is shown. The initial fast component or the "tonic" component that precedes the "clonic" component in the adult animal (Fig. 4) is here completely missing.

Discussion. The findings described above make it more comprehensible why previous attempts by many investigators to reproduce the "spike and wave" after-discharge without admixture of "fast" activity were followed by rather controversial results. The main reason for this seems to be the fact that these investigators have been working exclusively on adult animals. And so it was reported(2)

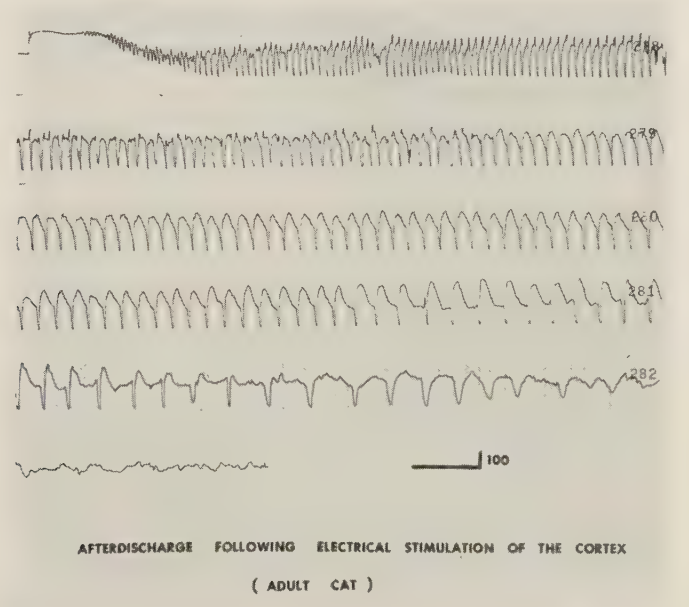


FIG. 4.

that stimulation of the medial thalamic structures may result in a "spike and wave" activity of large areas of the cortex. This activity was dependent on a stimulus frequency of about 3 per second. It was also found(3) that with metrazol sensitization and photic stimulation, repeated stimuli applied with a frequency of about 3 per second produce "spike and wave" formations in the cortex. However, the "spike and wave" discharges lasted only as long as the stimulation and have not been produced as a consistent self-maintained seizure activity after the withdrawal of the stimulus. If a self-sustained discharge occurred it consisted of "fast" spikes of the type seen during the "tonic" phase of a "grand mal" seizure.

The principles of development of the electrocorticogram of the cat indicate a tendency of the immature cortex to produce "slow" frequencies. This physiological electrical characteristic of immaturity is also manifested during the pathological state of an epileptic after-discharge. On this basis it is possible that the electrical picture of focal and non-focal "spike and wave" discharges so frequently found in the EEG of epileptic children may be explained by the readiness of the immature cortex to produce this type of discharge.

Summary. Metrazol and electrically induced seizure activity in the cortex of kittens at various stages of maturation reveals a relation of form and frequency of discharges to age. Focal and non-focal "spike and wave" discharges of frequencies seen in the EEG of the epileptic human infant and child may be reproduced in young animals. With increasing age spikes of faster frequencies replace this slower "spike and waves". The findings indicate the importance of electro-ontogenetic studies in investigations of the electrical patterns of epileptic seizures.

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Electrophoretic Mobility—Ionic Strength Studies of Proteins. I. Heterogeneity of Human Serum Albumin.* (21008)

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The moving-boundary method of electrophoresis as developed by Tiselius(1) offers a powerful tool for testing one aspect of the purity of proteins, *i.e.*, their homogeneity with respect to mobility(2). Such crystalline proteins as serum albumin(3) conalbumin, chymotrypsin, ribonuclease, etc.(4), B-lactoglobulin(5) and many other proteins were found to give a single moving boundary over a certain range of pH but *2 or more* boundaries under other conditions. By the technics of electrophoretic mobility, v.s. pH (3,6-10), prolonged electrophoresis(11,12), or electrophoresis in the presence of certain anions (13,14), human serum albumin showed 2 or more moving boundaries. Leutscher(3,6) carried out his original studies at pH 4.0 and with low ionic strength (.02) acetate buffer and reported separation of normal human serum albumin into 2 "components". Miller, *et al.*(9) also used acetate buffer at pH 3.9 but of high ionic strength (0.2, 90% NaCl) and reported separation of human serum albumin into *4* "components". It was our belief that this discrepancy in the number of boun-

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daries formed, as well as in their sharpness of resolution for quantitative analysis, could best be resolved by a careful, systematic study of the effect of variation of the buffer ionic strength on the electrophoretic mobility of purified human serum albumin. The importance of the ionic strength factor, *especially at low salt concentrations,* on the electrophoretic mobility of proteins has been stated by Svensson(15) as being of the same order of magnitude as that of pH. The experimental studies presented in this paper show that it is possible to separate human serum albumin, which shows a single boundary at pH 8.6, 0.1 ionic strength barbiturate buffer, into at least four distinct peaks at pH 4.0, ionic strengths 0.06 to 0.03, acetate-NaCl buffer, for the ascending boundaries.

A decrease in the serum albumin component at pH 8.6, 0.1 ionic strength is a characteristic of many chronic diseases, *e.g.,* multiple sclerosis(16), as well as of most acute diseases (17,18). Leutscher(6) has shown that for such diseases as nephrosis, or cirrhosis of the liver, a reversal of the normal ratio of the areas under the two peaks formed occurs at pH 4.0 and .02 ionic strength, acetate buffer. It is believed that the quantitative fractionation of human serum albumin into four or more components, as described here, by moving boundary electrophoresis may prove to be useful in the clinical study and evaluation of many diseases.

Experimental. In order to determine the electrophoretic mobility and homogeneity of the albumin† solution used in these studies, 2 ml of 25% solution are diluted to 10 ml with barbiturate buffer(20) (pH 8.6, 0.1 ionic strength) and then dialyzed in Visking tubing for a minimum of 5 hours in a mechanical dialyzer(21) against 400 ml of the buffer at 25°. The analysis was performed with the portable Aminco-Stern electrophoresis apparatus employing a previously published technic(16). Electrophoresis was carried out at 2 degrees and the pattern obtained was photographed and traced (Fig. 1a). In the experiments shown in Fig. 1b and 1c, the 5% serum albumin solutions were run electrophoretically in barbiturate buffer at pH 8.6, ionic strengths .02 and .005 respectively. A series of electrophoretic determinations were performed on an albumin sample diluted in the same manner but dialyzed against acetate-NaCl buffer(22) and pH 4.0 and at the ionic strengths shown in Fig. 2. The final pH of each buffer solution after dilution with distilled water was checked with a glass electrode and adjusted to pH 4.0 ± 0.02 pH units. The electrophoretic analyses were run exactly as described above except for changes in the field strength and duration of run as shown in Fig. 2a to 2h. The mobility of the leading component for each ionic strength given in Fig. 2 was calculated from the ascending pattern and the results obtained in these runs are plotted in Fig. 3. A number of other samples fractionated with Cohn's technic were obtained and showed the same splitting into 4 distinct peaks under our experimental conditions. In addition, an albumin sample was

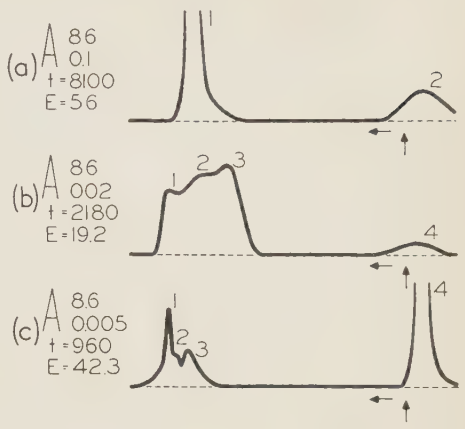

FIG. 1. Fractionation of 5% human serum albumin (A) solution; pH 8.6 barbiturate buffer, ionic strengths (a) 0.1, (b) 0.02, (c) 0.005. All conditions were kept constant except potential gradient (E = volts/cm) and duration (seconds) as noted for each diagram. Vertical arrows indicate starting positions. All photographs and diagrams in this paper are of the ascending boundaries. Peak 2 in Fig. 1a represents the classical protein-buffer salt (or delta) boundary.

† Albumin in these studies obtained from American National Red Cross through the courtesy of Dr. J. N. Ashworth. It is prepared by the ethanol-low temperature fractionation procedure of Cohn and his associates(19). It consists of 25% albumin solution and contains 0.02 M sodium caprylate and 0.02 M sodium acetyl tryptophanate as preservatives.

prepared from normal human serum by salt fractionation with 28% sodium sulfite by the procedure of Wolfson, *et al.*(23), the excess salt was removed by dialysis and the sample concentrated to about 2% albumin by evaporation in a cellophane bag. The results obtained for human serum albumin prepared in this manner and run at pH 4.0 and 0.03 ionic strength are shown in Fig. 4a. In order to make certain that the splitting of the albumin into a number of boundaries under our experimental conditions does not represent denaturation products or artefacts obtained at pH 4.0, the following series of experiments were performed: a) A 5% serum albumin sample was run electrophoretically in the manner described above at pH 4.0 and 0.03 ionic strength and the usual 4 peaks were obtained (Fig. 4b). The protein sample was withdrawn and the acetate buffer removed by overnight dialysis in cold running water. The solution was then dialyzed mechanically at 25° against repeated changes of barbiturate buffer (pH 8.6, 0.1 ionic strength) and rerun electrophoretically, giving a *single* peak as is shown in Fig. 4c. Satisfactory results were also obtained with the reverse experiment, *i.e.*, running the sample first at pH 8.6, 0.1 ionic strength and then at pH 4.0, 0.03 ionic strength to obtain the 4 boundaries. b) In an experiment performed as described in (a) above, the run was continued at pH 4.0, 0.03 ionic strength for 1 hour until 4 distinct boundaries were obtained. The polarity of the electrodes was then reversed and the same potential applied for the same time, until the pattern reformed into a single but less-sharp boundary. c) In a run performed as described under (a) above, photographs were taken every 15 minutes for a total of 4 exposures and the mobility of the leading component calculated (Fig. 4d).

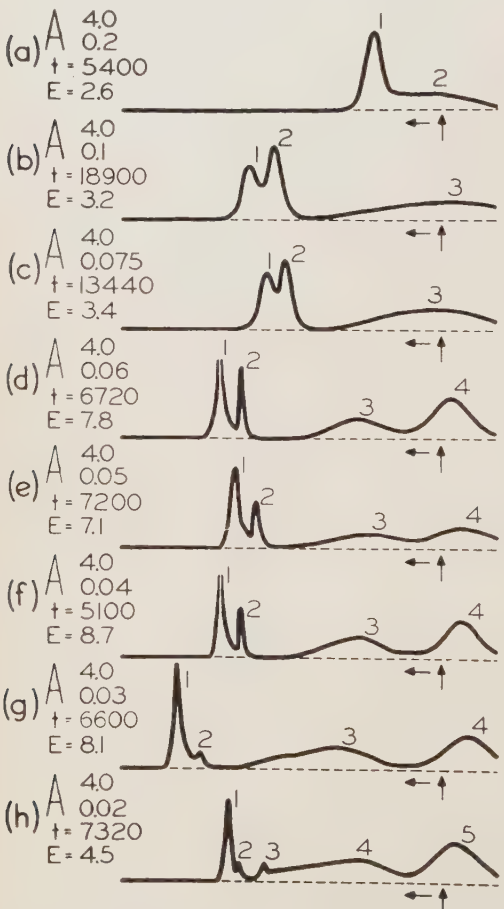

FIG. 2. Fractionation of 5% albumin solutions; pH 4.0 acetate-NaCl buffer, showing increase in number of boundaries with decrease in ionic strength of the buffer. Note change in relative areas in peaks labeled 1 and 2 with decreasing ionic strength.

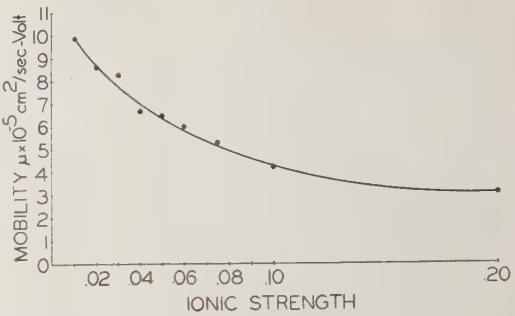

FIG. 3. Change in electrophoretic mobility of fastest moving boundary (peak 1) with increase in ionic strength of acetate-NaCl buffer.

Results. A) *Effect of ionic strength variation with barbiturate.* The ascending electrophoretic patterns obtained for 5% human serum albumin solutions at pH 8.6, barbiturate buffer, and at ionic strength of 0.1, 0.02 and 0.005, respectively, are shown in Fig. 1. These patterns provide experimental proof that sufficiently large variations of the ionic strength factor can be just as important in affecting the mobility relationships of a protein mixture as are the pH changes previously

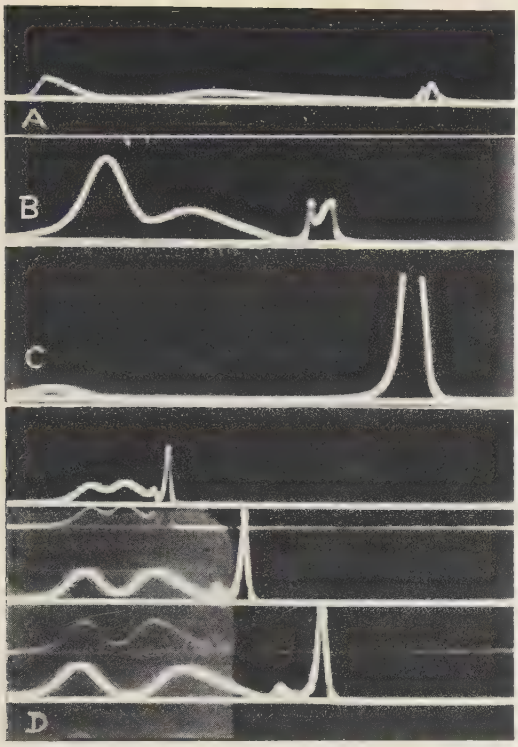

FIG. 4. (a) Electrophoretic pattern of normal human serum albumin prepared by sodium sulfite (28%) fractionation of serum. Acetate-NaCl buffer pH 4.0, ionic strength 0.03. (b) American Red Cross serum albumin prepared by ethanol-low temperature fractionation. Electrophoretic subfractionation in acetate-NaCl buffer, pH 4.0, ionic strength 0.03. (c) Recovered solution from (b), redialyzed and rerun electrophoretically in barbiturate buffer pH 8.6, ionic strength 0.1. (d) Sequence photographs (every 15 min.) of the subfractionation of 5% albumin solution in acetate-NaCl buffer, pH 4.0, ionic strength 0.03. Solution was recovered from a previous run in barbiturate buffer, pH 8.6, ionic strength 0.1.

investigated(2-10). It can be noted that while at an ionic strength of 0.1 (Fig. 1a), the albumin appears as the usual sharp, single boundary with a calculated descending mobility of -6.8×10^{-5}cm^2 per sec. per volt, a 5-fold decrease in the ionic strength of the buffer to 0.02 (Fig. 1b) gives the "shoulder" type of boundary found by Miller, *et al.*(9) at pH 3.9, 0.2 (90% NaCl) ionic strength, acetate buffer. When the ionic strength of the barbiturate buffer at pH 8.6 is decreased 20-fold to 0.005, the further separation of the serum albumin into a number of separate boundaries is clearly evident as is seen in Fig. 1c. In these runs all other variables were kept constant except those noted in Fig. 1.

B) *Effect of ionic strength variation with acetate buffer.* The acetate buffer, pH 4.0, used in these runs is that proposed by Miller and Golder(22) in which 90% of the ionic strength is furnished by the NaCl content of the solution. The buffering capacity of this acetate buffer is less and its conductivity greater than the acetate buffer of the same pH and ionic strength employed by Leutscher (3,6) in his studies of albumin fractionation. This difference in the acetate buffers used by these investigators could serve to explain the fact that with Miller's buffer at pH 4.0, *0.20* ionic strength, the two boundaries obtained by us and the relative areas under them, correspond closely to those found by Leutscher (3) for his acetate buffer at pH 4.0, *0.02* ionic strength, although the boundaries are somewhat sharper in the latter case. As the ionic strength of the acetate-NaCl buffer at pH 4.0 is decreased from 0.20 to 0.02, the number of distinct boundaries formed increases from 2 to 5 as seen in Fig. 2. Only ascending boundaries are shown in these tracings. In general we have tended to designate with numbers only those boundaries which are both distinct and capable of quantitative measurement of their areas. A decrease in the ionic strength under our experimental conditions, as is clearly evident in Fig. 2, also results in a change in the relative areas under the two fastest moving ascending boundaries, *i.e.*, peaks 1 and 2. As the ionic strength decreases from 0.075 to 0.02, there is a continuous increase of the area under peak 1 and a corresponding decrease of the area under peak 2. According to the Debye-Huckel extension of the diffuse "double layer" theory of Gouy (24), as the ionic strength is increased, the layer becomes thinner and the mobility decreases. That this is indeed the experimental findings for the fastest moving ascending boundary is shown graphically in Fig. 3 where the mobility decreases continuously toward an asymptotic level as the ionic strength is varied from 0.01 to 0.2. All other experimental conditions were kept constant in these runs except for those noted in Fig. 2a to 2h.

C) *Effect of time of dialysis on electro-*

phoretic patterns obtained. Few studies(14, 25,26) have been made of the ionic strength-mobility relationships of proteins over a wide range of values for a particular buffer system although pH-mobility studies of proteins appear quite frequently in the literature. Except for Luetscher's(3) work with serum albumin little work has been done in the low ionic strength range, *i.e.,* below 0.05. One of the reasons for the lack of such studies has been pointed out by Armstrong and his coworkers (14). They state that at the extremes of the ionic strengths studied, *i.e.,* above 0.3 or below 0.05, variability in resolution of the individual diagrams proved too great to permit the evaluation of the influence of the ionic strength factor. Dialysis of protein solutions is usually carried out against the buffer to be employed for periods of 12 to 48 hours at 4-5° sometimes with several changes of the buffer solution used, (9,27). While this may be adequate for ionic strengths from 0.05 to 0.2, albumin solutions in low ionic strength buffers require a dialysis period of 72 hours or longer at 4° for reproducible results. For the sake of convenience it is preferable to use a mechanical dialysis set-up as recommended by Reiner and Fenichel(21) for a minimum of 5 hours at room temperature. Duplicate patterns thus obtained with a 5% protein solution in 0.03 ionic strength, acetate-NaCl buffer, run under identical conditions but several days apart, are practically superimposable and give almost identical measurements of the areas under the various peaks.

D) *Effect of variation of albumin concentration.* It would be advantageous for clinical studies in protein metabolism to be able to work at as low an albumin concentration as would be consistent with good reproducible results for the areas under each of the 4 peaks. A series of determinations were then run as above, at pH 4.0, 0.05 ionic strength, in which the albumin concentration was varied from 0.5 to 5.0%. The results obtained in such runs, which are not shown here, indicate that clear-cut separations into 4 distinct peaks are obtained at protein concentrations between 1.0-5.0%.

E) *Effect of method employed for albumin separation.* Experimental evidence that the observed boundaries for human serum albumin are protein in nature, and not artefacts, is provided by the work of Hoch and his coworkers(12,28) and by Reinhold and Gilman (10). Further evidence in this direction is provided by running 5% albumin in acetate-NaCl buffer ionic strength 0.1, pH 4.0; withdrawing the sample after its fractionation, as in Fig. 4b, and rerunning after suitable dialysis in barbiturate buffer pH 8.6, ionic strength 0.1. Under the latter conditions the albumin migrates as a single boundary as seen in Fig. 4c. Opposite results were obtained by the reverse procedure, that is, running the albumin sample in barbiturate buffer at pH 8.6 first. Similarly the reversible boundary-spreading test(2) while indicating the heterogenity of serum albumin also shows the separation of the albumin into 4 or more components to be reversible since the original boundary can be reformed by changing the polarity of the electrodes. This would indicate that the forming of multiple boundaries with serum albumin solution is not due to either denaturation or irreversible dissociation into smaller molecular weight units.

The constancy of the mobility of the leading boundary with time would also provide evidence against any change in the surface charge density of the protein molecules such as would occur with either denaturation or dissociation of the molecule. In addition the photographs shown in Fig. 4d, illustrate the changes of the electrophoretic pattern with time, until maximum development into separate boundaries occurred, under the given experimental conditions.

Discussion. The use of the word "component", with its implied meaning of a distinct molecular species, has been avoided. Instead the terms "boundaries" or "peaks" have been employed interchangeably. This usage more accurately describes the phenomena which occur during the electrophoresis of proteins and which is observed and recorded photographically by the schlieren lens system(27). Provided no false boundaries are present, the number formed with the electrophoretic method must be considered to be a *minimum* estimate of the number of distinct molecular species (or components) present in a mixture.

The criteria for the purity of a protein has been reviewed by Li(29) who states that the more satisfactory evidences are the physicochemical properties such as solubility, electrophoresis, etc. As previously mentioned few purified proteins, if any, meet even these tests of homogeneity. These experimental findings had led Haurowitz(30) to assert that it is possible that completely pure proteins do not exist, and that each of the so-called "pure" proteins is in reality a mixture of very similar protein molecules. The experimental work reported here for human serum albumin would tend to support this viewpoint.

At ionic strengths of 0.01, or less, the patterns obtained do not give the clearly resolved, measurable peaks formed at the higher ionic strengths. Since we are more concerned with the *quantitative* rather than the qualitative aspects of albumin fractionation, we arbitrarily chose an ionic strength of 0.03 (pH 4.0 acetate-NaCl buffer) which gives four clearly resolved and measurable boundaries, as the most convenient experimental conditions for most of our studies.

As stated by Longsworth(28) and Alberty (2) "ideal" electrophoresis of protein mixtures should be carried out at low protein concentrations and with high ionic strength buffers so that the protein itself would carry a negligible charge. In actual practice it is not possible to carry out electrophoresis under "ideal" conditions and as a result there are always differences between the ascending and descending patterns. While the conditions under which our experiments would run would appear to be "non-ideal", a number of mitigating factors exist. For example, most of our experimental work was performed at pH 4.0 at which the albumin (isoelectric point, pH 4.5) carries only a slight positive charge. In addition, reduction of the albumin concentration to 1%, or less, still gives similar patterns as were obtained at a 5% protein concentration. However, it is an experimental fact that human serum albumin can be fractionated electrophoretically into a number of peaks in low ionic strength buffers whether the protein carries a large negative charge (pH 8.6, .005 ionic strength, barbiturate buffer) or a small positive charge (pH 4.0, .03 ionic strength, acetate-NaCl buffer). We have preferred to use the latter conditions for the same reasons that most investigators working with plasma proteins prefer to use barbiturate buffer at pH 8.6 rather than phosphate buffer at pH 7.7, namely, that additional ascending boundaries are obtained and there is better resolution of these boundaries for quantitative purposes.

It should perhaps again be emphasized that Miller's(22) acetate-NaCl buffer is *not* the same as the acetate buffer employed by Leutscher(3,6) in his studies, although both can be made up to an equal ionic strength, *i.e.*, 0.02. The fact that the addition of NaCl to a buffer to increase the ionic strength causes changes in the electrophoretic patterns has been previously cited by other investigators (13,31). In fact, Armstrong, *et al.*(14), found that a synthetic mixture of albumin and beta-globulin which gave one boundary with sodium diethylbarbiturate buffer, 0.1 ionic strength, was separated readily by electrophoresis in sodium diethylbarbiturate-sodium caprylate buffer, 0.1 ionic strength. By combining the experimental conditions of Luetscher(3) with the acetate-NaCl buffer of Miller and Golder(22) we have been able to verify Miller's(9) findings that serum albumin contains at least 4 "components" and that the results obtained are highly reproducible.

Electrophoretic phenomena, similar to our observations on serum albumin, at pH values just below the isoelectric point have been reported by Longsworth and Jacobsen(32) for bovine serum albumin and B-lactoglobulin. These authors suggest that under these conditions the equilibria prevailing in the body of the protein solution are being continually adjusted in the boundary layers as electrophoretic separation takes place. It is also a well known fact that serum albumin binds readily with large molecules, *e.g.*, lipoproteins (14) and bilirubin(11), and with small ions (33). The authors prefer to withhold their own interpretation of these phenomena pending further experimental work. The electrophoretic separation and the quantitative analysis of the substances present under each peak seen in the electrophoretic patterns is now in progress in this laboratory. Some pre-

liminary results already obtained for other proteins, *e.g.*, human gamma globulin, indicate that we are dealing with a generalized phenomena in which additional boundaries can be obtained by means of electrophoretic separation just below the isoelectric point of the protein in the presence of suitable low ionic strength buffers. The extension of the technic of sub-fractionation described here to clinical studies of isolated protein fractions from sera of diseased cases such as nephrosis(6), cirrhosis(6), multiple sclerosis, cancer, etc., may provide useful information about their protein metabolism.

Summary and conclusions. 1. The ionic strength-electrophoretic mobility relationship of human serum albumin was studied at pH 4.0 in acetate-NaCl buffer over a wide range of ionic strength values. 2. A 5% albumin solution which gave a single boundary at pH 8.6 in barbiturate buffer, 0.1 ionic strength, showed separation into a number of ascending boundaries at an ionic strength of 0.005. 3. Similarly at pH 4.0 in acetate-NaCl buffer, the number of ascending boundaries increases from 2 to 5 as the ionic strength is decreased from 0.2 to 0.02. Under these conditions, the area under the fastest moving peak increases continuously at the expense of the slower-moving peak adjacent to it. 4. Using an ionic strength of 0.03 (pH 4.0) as a standard condition, since it gives 4 clearly resolved boundaries, various other factors which may influence the electrophoretic pattern were studied, *e.g.*, protein concentration, method of preparation of albumin fraction, time of run, and technic of dialysis used. 5. Reproducible values for the areas under each of the 4 peaks were obtained at protein concentrations between 1 to 5% and with mechanical dialysis for 5 hours or longer at 25° against the acetate-NaCl buffer, 0.03 ionic strength. 6. Preliminary work would indicate that the electrophoretic sub-fractionation of other proteins or protein fractions, *e.g.*, gamma globulin, is also possible and that this behavior may be part of a more generalized phenomena. 7. The possible explanation of these phenomena await quantitative analysis of the components present under each observed peak. However, evidence is presented that boundaries probably represent protein components and not artefacts.

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Alteration in Pentose Content of Chick Embryos Infected with Japanese Encephalitis Virus. (21009)

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The developing chick embryo has been used as a medium for the cultivation of a wide variety of obligate intracellular parasites, but the host-parasite relationship involved has received comparatively little attention. Since the embryo undergoes marked changes in size and composition during the growth period of the infectious agent, it necessarily constitutes a complex and dynamic medium.

The nucleic acids of the embryo are constituents of particular interest because of the direct correlation between ribonucleic acid concentration and the rate of protein synthesis (1-3). The invasion of unicellular hosts (*E. coli*) with bacteriophage has been shown by Cohen(4,5) to involve an inhibition of ribonucleic acid synthesis, whereas the synthesis of desoxyribonucleic acid proceeds at an essentially normal rate. Our observations have indicated that infection of chick embryos with the virus of Japanese encephalitis induces changes in tissue composition of comparable magnitude and in the same direction as those found in bacterial cells invaded by phage.

Materials and methods. Embryonating eggs of the White Leghorn were selected at random from several large and representative batches. All of the eggs used contained actively motile embryos with pronounced peripheral blood vessels visible upon candling. The eggs were incubated at 38°C and a relative humidity of 40 to 60%. Age of the embryo was designated as the number of days of incubator life. The embryos subsequently dying in the course of the experimental work were assumed to be dead when examination by candling disclosed that spontaneous motion of the embryo had ceased and the peripheral blood vessels had retracted. Infection with Japanese encephalitis virus (Nakayama strain, egg-adapted) was accomplished by inoculation of the chorio-allantoic membranes of 10-day-old embryos with 0.1 ml doses of a 1% suspension of infected chick-embryo tissue in phosphate buffered saline solution, pH 7.8. Twenty to 100 inoculated eggs were used in each experiment, with an equal number of control eggs from the same group. The eggs were maintained at 35°C and examined by candling at 4-hour intervals. All embryos which died less than 48 hours after inoculation were discarded. When the mortality ratio exceeded 20% (generally 60 to 72 hours after inoculation) both the infected and the normal

TABLE I. Pentose and Desoxyribonucleic Acid Concentrations in Infected and Normal Chick Embryo Tissues. 7 experiments.

	Pentose (mg % wet tissue)				Desoxyribonucleic acid (mg % wet tissue)			
	Infected embryos			Normal embryos	Infected embryos			Normal embryos
	I Dead	II Living, hemorrhagic	III Living, non-hemorrhagic	IV	I Dead	II Living, hemorrhagic	III Living, non-hemorrhagic	IV
	71	92	105	131	217	241	229	237
	37	92	96	99	166	210	212	208
	50	93	94	89	200	214	216	212
	60	70	100	104	233	202	196	220
	47	60	109	112	170	181	220	195
	49	80	101	106	176	219	201	204
	48	58	71	92	198	201	211	195
Mean	51.7	77.9	96.6	104.7	194.3	209.7	212.1	210.1

control eggs were harvested. The infected embryos were divided into three groups at the time of harvest: I) embryos dead 4 hours or less, II) embryos living but profusely hemorrhagic, and III) embryos living and not hemorrhagic. Group IV consisted of the normal embryos from the control group. The embryos were removed from the shells and allowed to drain free of egg fluids. After weights were taken, the embryos were macerated in a Waring Blendor in a solution of sodium chloride (0.1 M), sodium citrate (0.1 M), and sodium desoxycholate (0.2%) to prepare homogenates containing 20% (wet weight) of tissue. This procedure was based on the technic devised by McCarty and Avery (6) and modified by Schneider(7). After standing for 2 hours at 4°C, the homogenate was deproteinized by shaking for 10 minutes with ½ volume of a mixture (after Sevag(8)) of chloroform (4 volumes) and isoamyl alcohol (1 volume). Separation of the phases was accomplished by centrifugation for 1 hour at 2°C at a mean acceleration of 2000 g. The supernatant aqueous phase was removed by siphonation. The chloroform phase was discarded. The mass of insoluble material at the interface was resuspended in a small volume of the disrupting fluid and re-extracted. Aliquots of the pooled aqueous phase were analyzed without further purification. Duplicate or triplicate determinations were made in all cases. It was demonstrated that the aqueous extracts as prepared contained more than 99% of the total extractable pentose and DNA.

Total pentose (excluding desoxypentose) was estimated by the orcinol reaction of Kerr and Seraidarian(9) with reference to a standard solution of d-ribose. The specificity of the orcinol reaction for pentose in the presence of other sugars has been verified by Brown(11) and Fernell and King(12). Seventy to 85% of the pentose measured is accountable as ribonucleic acid (RNA) on the basis of its contribution to the optical density of the solution at 258 mμ. The results of the analyses were reported in terms of pentose, however, to avoid the introduction of errors in conversion. Desoxyribonucleic acid (DNA) was estimated by a modification (10) of the Dische diphenylamine reaction. The reference standard was a solution of sodium desoxyribonucleate.*

Results. The pentose and DNA concentrations found in the infected and normal embryos in 7 experiments are shown in Table I. A variance analysis of the data by a modification of the method of Snedecor(13) is summarized in Table II. Pentose concentration in the infected embryos was significantly lower than that of the normal embryos. The diminution in pentose concentration is greatest in the dead embryos, and the living hemorrhagic embryos were lower in pentose content than the non-hemorrhagic infected embryos. DNA concentration was not significantly different among any of the groups.

Discussion. Infection of 10-day-old chick embryos with Japanese encephalitis appears to

* Obtained from Nutritional Biochemicals Corp.

TABLE II. Variance Analysis of Data.

Source of variation	DF	SS	MS	F
Pentose				
I II III vs IV	1	4,517	4,517	39.3*
I vs II III	1	5,881	5,881	51.1*
II vs III	1	1,226	1,226	10.6†
Between exps.	6	2,146	358	3.1‡
Interactions:				
(I II III vs IV) × exps.	6	514	86	
(I vs II III) × exps.	6	734	122	
(II vs III) × exps.	6	823	137	
Error	(18)	(2,071)	115	
Total	27	15,842		
Desoxyribonucleic acid				
Between groups	3	1,432	477	2.2
" exps.	6	4,025	671	3.1‡
Error	18	3,874	215	
Total	27	9,331		

Level of significance:
* 0.1% † 1% ‡ 5%

effect a reduction in pentose content of the tissues prior to death of the embryo. This alteration in tissue composition is demonstrable by analysis of the entire body of the embryo, indicating that most or all of the cells of the body are affected. The mechanism of the alteration has not been elucidated. Since the virus of Japanese encephalitis is pantropic in the chick embryo(14), it is possible to infer that the invasion of virus particles has impeded aerobic glycolysis in the individual cells. The phenomenon would then be parallel to what happens in bacteria invaded by phage(4,5). However, the organized structure of the embryo could sustain functional defects on a higher physiological level which would elicit the same result. A respiratory insufficiency in the dying embryo, for example, might force the individual cells to abandon aerobic glycolysis. A comparative study of embryos dying from other causes will be required for the elucidation of the observations reported. It will also be of interest to determine the correlation between the metabolic state of the embryo and its virus content.

Summary. The tissues of chick embryos dead or dying following infection with the virus of Japanese encephalitis are significantly lower than normal in pentose concentration but no significant differences in desoxyribonucleic acid concentration were demonstrated. The factors responsible for the decrease in pentose have not been elucidated.

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Tissue Distribution of Boron Compounds in Relation to Neutron-Capture Therapy of Cancer.* (21010)

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The promising direct application of the atomic pile to the treatment of patients with malignant brain tumors, investigated by Farr, Sweet, *et al.* at Brookhaven National Laboratory during the last two years, has focussed attention on a number of problems relating to the biological behavior of boron compounds. This mode of cancer therapy is based on the fact that certain nuclides, notably boron-10, disintegrate on capturing a neutron with the release of high energy heavy particles.† By virtue of their charge, their internal origin and other factors, these particles appear to have a therapeutic effectiveness some 10 to 20 times that of equivalent energy as x-rays. Moreover, their relatively large mass permits them even at these high energies to travel an average of only 10-15 μ in tissue. This means that the lethal disruptive effect from the disintegration of that atom is confined to a zone only twice the diameter of a red blood cell. The beauty of this concept, first proposed on theoretical grounds by Kruger(1), and later experimentally by Zahl, Cooper and Dunning (2), resides in the fact that in boron-neutron treatment biologically effective radiation is induced only at the site of interaction of two components which are in themselves relatively innocuous, whereas in the usual administration of radioactive isotopes for therapeutic purposes, radiation is delivered to whatever parts of the body the isotopes may reach, often concentrating in such undesired places as normal liver and bone marrow. A neutron beam on the other hand can be directed to any desired portion of the head or body. Its practical usefulness, however, requires a sufficient concentration of boron in the cancer tissue, a favorable ratio between this concentration and that of the surrounding normal tissues and, finally, a high enough flux of *thermal* neutrons at the cancer site. Conger and Giles(3) presented the first quantitative evaluation of the biological effects of this neutron-boron reaction in plants, and their lucid exposition led Sweet(4) to propose that neutron-capture therapy might be applied with unique advantage to the treatment of brain tumors. He, with Javid(5) demonstrated, after the intravenous injection of borax, useful concentration ratios between malignant tumors and normal brain of some 3 or 4 to 1. This paved the way for the actual clinical trial of neutron-capture therapy with boron-10, which has thus far been carried out in 10 patients for a total of 21 irradiations at the Brookhaven National Laboratory reactor(6).

These early studies have brought up the following fundamental questions: 1) Is tissue concentration of borate, and hence effective radiation during neutron bombardment, proportional to the injected dose? 2) How is borate partitioned between the extracellular and intracellular tissue compartments? (The latter favors greater cell nucleus disruption by the secondary particles.) 3) Does the distribution of borate suggest that neutron-capture therapy may be applicable to neoplasms other than brain tumors?

Methods. In other communications it was demonstrated that pure-strain mice bearing transplantable malignant brain tumors provide a useful means of assaying distribution and biological behavior of various radioactive and stable isotopic compounds, and that such information can be carried over with reasonable confidence to predict the behavior of the

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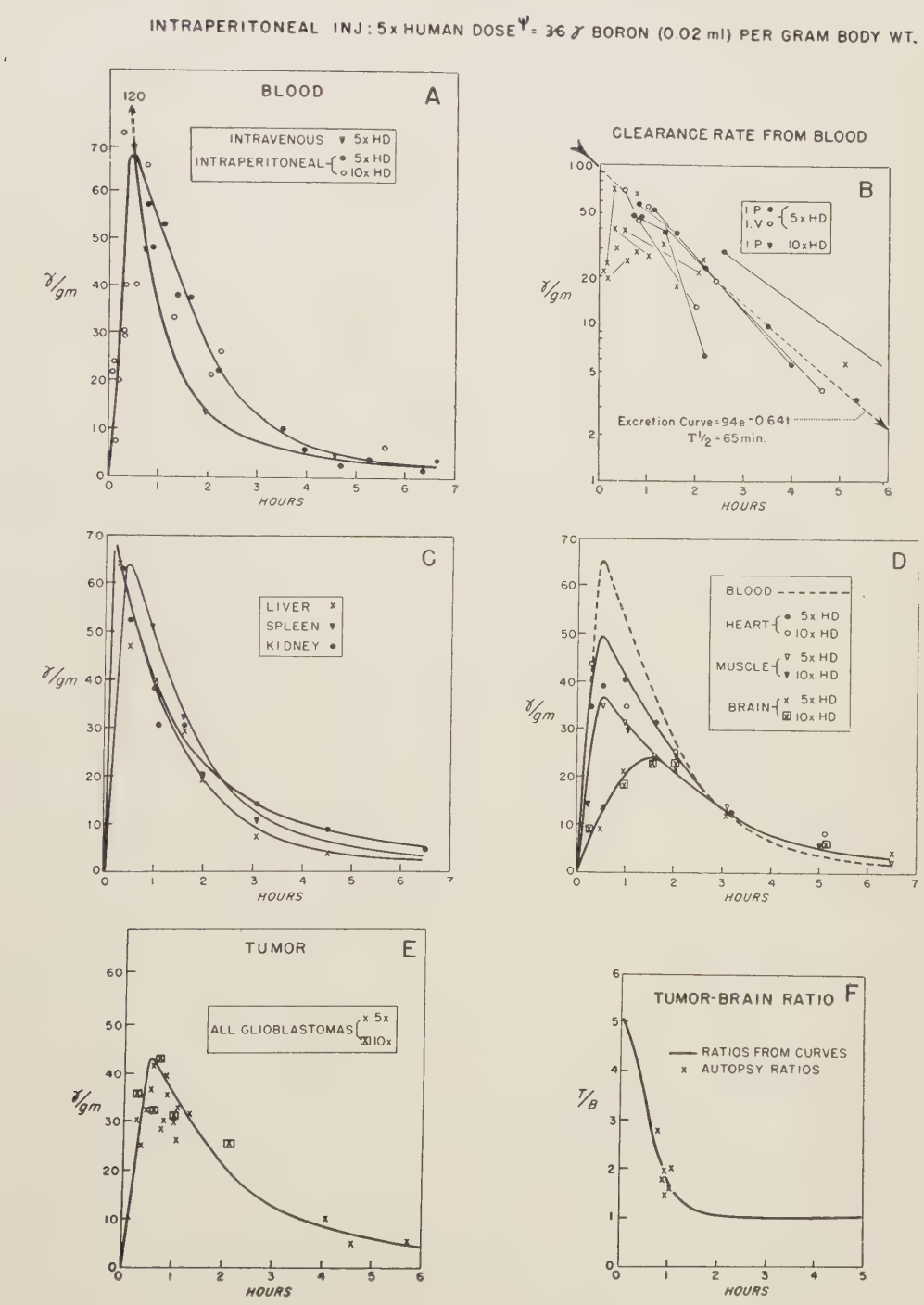
† Expressed as a nuclear equation:

$${}_5B^{10} + {}_0n^1 = [{}_5B^{11}] = {}_3Li^7 + {}_2\alpha^{4++} + 2.79 \text{ Mev}$$

In this interaction, boron has a capture cross section of 3900 barns. The unstable B^{11} disintegrates in less than a microsecond into an ionized lithium nucleus and an alpha particle, and the 2.79 Mev of free energy is divided between them as kinetic energy approximately in the inverse ratio of their masses.



* DATA EXPRESSED AS MICROGRAMS (γ) BORON PER GRAM WET TISSUE
Ψ SEVERAL MICE RECEIVED 10x HD; THEIR TISSUE LEVELS PLOTTED TO 1/2 SPACE

FIG. 1.

same agent in human brain and brain tumors (7,8). This mouse-assay technic has been applied to the present studies of boron compounds, and details of the experimental method may be found in these two reports. The mouse brain tumors used in our experiments were induced by implantation of 20-methylcholanthrene according to the method of Seligman and Shear(9) in the laboratory of Dr. Harry Zimmerman, and they have passed through more than 50 generations of subcutaneous transplantations in mice of the same strain. We have used 2 types classified by Zimmerman as glioblastoma and astrocytoma. Boron has been studied as borate in the form of borax, and since there is no radioisotope of boron suitable for tracer work, the data have been obtained by chemical analysis, using the method of Ellis, Zook and Baudisch (10). The borate curves for man, presented for comparison, were drawn from a replotting of data by Sweet and Javid(5). Their studies were all done during operations on patients with highly malignant brain tumors, when it is possible to obtain small samples of brain and tumor at intervals after injection from the area of the brain being resected, without compromise to the patient.

Results. Body distribution and excretion. The distribution characteristics of borate administered intraperitoneally in mice are shown in Fig. 1. Absorption and distribution to the tissues are very rapid, and by 30 minutes all organs, except brain, have reached a peak. The various organs form a family of similar curves such that the faster the rise in concentration, the higher the peak and the earlier its occurrence. Thereafter the level in blood and tissues falls off rapidly; and since there is apparently no tissue reservoir soaking up large amounts of borate at this time, this sharp decline can reasonably be attributed to excretion. After roughly three hours all the tissues, including the brain, appear to be in equilibrium and losing borate at about the same rate, suggesting that their levels are now being controlled largely by the excretory mechanism. This is well illustrated by the tumor-brain ratio curve (Fig. 1F): from its initial value of 5 or 6 to 1 it falls sharply to around 2:1 by one hour, and remains about 1:1 from two

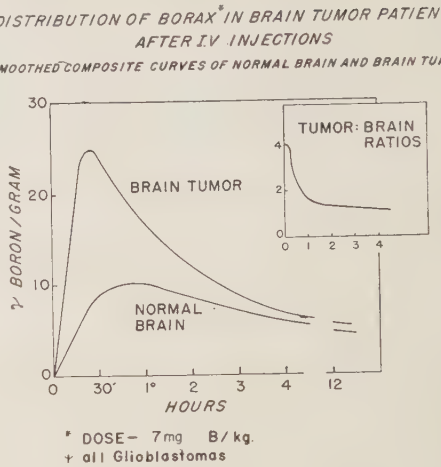


FIG. 2.

hours to the end of the curve. Having observed the rapidity and importance of excretion, we made an indirect calculation of its rate. Fig. 1B is a semilog plot of all our blood concentration data in mice, with lines connecting serial samples from the same mouse. After the peak is reached, the decline in blood levels (followed also by the tissue levels) is very nearly linear, indicating that excretion proceeds roughly as a simple exponential function; and from the average of the various slopes, the excretion half-time is determined to be 65 minutes.‡ One mouse, sacrificed two hours after injection, was found to have a full bladder, a fortuitous situation in view of the usually irresponsible excretory habits of these creatures. This enabled us to determine that the concentration of borate in the urine was 20 times the 2 hour plasma concentration and 5 to 10 times the mean plasma level during the 2-hour period. From this we judge that borate is cleared by the kidney as a "low-threshold substance" in a class with urea and sulphate, tubular reabsorption of which is primarily by passive diffusion.

Comparison with human data. In Fig. 2 the distribution of borax between tumor and brain in man is shown for comparison. A remarkable similarity in behavior may be noted: the peaks occur at about the same

‡ It will be seen later on more refined analysis that the disappearance curve has two exponential components.

TABLE I. Tissue Levels of Borate* vs. Dosage in Mice.

Mouse #	Time	Dose†	Route	Blood		Heart	Muscle	Brain	Liver	Spleen	Kidney	Dose ratio‡	Avg tissue ratio‡
				Plasma	RBC								
V-14	31′	.25X	S.C.	1.8	1.7	1.6	—	.3	1.6	—	—	.05	.03
15	35′	.6 X	”	5.0	4.0	6.3	—	2.6	5.7	—	6.4	.12	.16
17	30′	2.0 X	”	14.2	15.0	15.4	—	3.2	13.0	—	13.0	.40	.33
S.C.§	*32′*	*5.0 X*	*I.P.*	*68*		*45*	*42*	*14*	—	—	—	—	—
V- 3	70′	.25X	I.P.	.7	1.0	1.0	—	.6	.8	—	1.5	.05	.03
20	65′	.6 X	”	2.6	2.9	3.4	—	2.2	3.0	—	3.6	.12	.095
6	65′	2.0 X	”	8.1	7.0	7.8	—	5.4	7.9	—	9.8	.40	.25
S.C.	*65′*	*5.0 X*	”	*47*		*38*	*32*	*21*	*37*	—	*37*	—	—
II-5	62′	10.0 X	”	54		70	61	36.2	—	—	—	2.0	1.8
V- 9	2h	.6 X	I.P.	2.4	1.6	1.9	—	—	2.4	—	2.4	.12	.09
S.C.	*2h*	*5.0 X*	”	*26*		*35*	*22*	*21.5*	*21*	*24*	*24*	—	—
II-4	2h	10.0 X	”	42.4		50	50.2	44	—	—	—	2.0	2.0
V- 2	4.5h	.6 X	I.P.	.54	.88	.51	—	.56	.48	—	1.0	.12	.12
S.C.	*4.5h*	*5.0 X*	”	*4.5*		*6.0*	*6.5*	*5.5*	*3.5*	—	*7.0*	—	—
S.C.	*6.7h*	*5.0 X*	*I.P.*	*2.8*		*2.0*	*2.6*	*4.2*	*3.0*	*3.0*	*4.5*	—	—
I-42	6.6h	15.0 X	”	4.5		5.0	5.5	5.1	4.5	4.5	8.2	3.0	1.8
I-43	6.8h	20.0 X	”	4.4		3.1	3.3	4.5	3.5	2.6	—	4.0	1.4

* Conc. expressed as γ boron/g wet tissue.

† Dosage factors are in terms of human dose of 70 μg borax (7.1 γ boron)/g body wt at operation, used in studies by Sweet and Javid.

‡ Dose-ratios and tissue-ratios are all compared to 5X human dose used in obtaining standard distribution curves in mice; the figures for brain have not been included for the first 30 min. in obtaining average tissue ratios.

§ S.C. = Standard curve for mice. (Whole blood data used.)

TABLE II. Distribution* of Borate in Normal and Nephrectomized† Mice.

	Mouse	Dose‡	Route	Time	Plasma	RBC	Heart	Brain	Liver	Kidney
A:	V-14	.25X	S.C.	31′	1.8	1.7	1.6	.3	1.6	N
	15	.6 X	"	35′	5.0	4.0	6.3	2.6	5.7	6.4
	16	.6 X	"	34′	4.6	5.2	5.5	2.4	6.0	N
	17	2.0 X	"	30′	14.2	15.0	15.4	3.2	13.0	13.0
	18	2.0 X	"	30′	15.3	14.4	13.4	5.5	14.4	N
B:	V- 8	.25X	I.P.	2h	1.4	1.4	.8	.5	1.0	N
	10	.6 X	"	2h	4.6	3.8	—	3.4	4.6	N
	12	2.0 X	"	2h	12.8	12.7	10.3	9.8	13.0	N
C:	V-16	.6 X	S.C.	34′	4.6	5.2	5.5	2.4	6.0	N
	10	.6 X	I.P.	2h	4.6	3.8	—	3.4	4.6	N
	1	.6 X	"	4h	5.3	4.7	4.9	3.7	5.0	N

* Conc. expressed as γ boron/g wet tissue.
† Nephrectomized mice indicated by N under "Kidney."
‡ Dosage factors refer to human dose of 70 μg borax (7.1 γ boron)/g body wt.

time, the tumor-brain ratio curves are nearly identical, and the rate of excretion is roughly parallel. There is one important discrepancy, however, which requires explanation. Whereas the curves for mice are based on a dose 5 times that used in man, their tissue levels are only 2 to 2½ times those seen for brain and tumor in man. From question (1) above the reader will readily understand the importance of this discrepancy to the calculation of tissue levels and radiation dosage during neutron-capture therapy.

Tissue levels vs. dosage. To test this relationship further, the ratio of tissue levels was studied in a series of mice given doses varying from 0.25 to 20 times the standard dose used by Sweet and Javid in patients at operation. The data, presented in Table I, indicate that tissue levels are in fact roughly proportional to dosage over the range from 0.25 X to 10 X for at least the first two hours, and that with 0.6 X and 5 X doses they are proportional up to 4.5 hours. (Only in the 15 X and 20 X doses at 6.7 hours is there serious disagreement—to be discussed later.) The 10 to 20% differences seen between the dose-ratios and the average tissue-ratios are within the experimental error of the method. Since in boron-neutron treatment, irradiation is carried out during the first hour, these data indicate that the concentration in brain and tumor may be calculated with confidence from the data of Sweet and Javid multiplied by the appropriate dosage factor. To test the effects of excretion on the proportionality of tissue levels and dosage, we did bilateral nephrectomies on a series of mice and carried out a parallel distribution study in them and a group of their normal confreres. The nephrectomized mice behaved with normal vigor and curiosity during the 24 hours they were studied and seemed to differ from the normal controls only in their abstinence from food and water. In order that they might not be jeopardized, they were given doses closer to the safe levels used in patients at operation rather than the 5-fold dose used in the mice of our control experiment (Fig. 1).

In Table IIA, tissue levels in normal and nephrectomized mice measured 30 minutes after injection of three dosage levels are compared. At this early time, both groups are seen to have about the same tissue levels for the same dose, and in both groups tissue levels and dosage are directly proportional. That this rough proportionality persists even when excretion stops is seen in Table IIB where 3 dosage levels are compared at 2 hours in nephrectomized mice.

In Table IIC, which shows tissue levels at varying times, but with the same dosage in nephrectomized mice, it is seen that equilibrium has been reached in heart, liver and red cells by 30 minutes, whereas brain continues to rise during the entire four hours. Mouse No. 16 was injected subcutaneously. Since this did not impair its tissue levels, which if anything were a little higher, it is concluded that borate is rapidly absorbed by all parenteral routes. The above studies indicate that up to 30 minutes, rates of absorption and distribution are the crucial factors determining

TABLE III. Distribution* of Borate at Equilibrium in Nephrectomized Mice.

Mouse	Dose†	Route	Time	Plasma	RBC	Brain	Heart	Liver
14	.25X	S.C.	31′	10	9.4	1.6	9.0	9.0
16	.6 X	"	34′	10.6	11.8	5.4	—	13.6
18	2.0 X	"	30′	10.0	9.4	3.6	8.7	9.4
10	.6 X	I.P.	2h	10.5	8.6	7.7	—	10.5
1	.6 X	"	4h	12.0	10.7	8.4	11.2	11.4
		Avg		10.6	10.0		9.7	10.8

* Conc. expressed as γ boron/g. Tissue levels for each mouse uniformly normalized by appropriate factor to a dosage of 10 γ/g.

† Dosage expressed in terms of standard human dose of 70 μg borax (7.1 γ boron)/g body wt.

the peak concentrations. They soon yield, however, to the dominant effects of excretion, which is apparent from the much lower levels seen in normal mice 30 minutes later (c.f. the 65-minute group of Table I). Since the discrepancy between mice and man resides fundamentally in the peak concentrations occurring at about 30 minutes, it seems likely from the above that the different routes of administration constitute the responsible factor. Since the tumor concentration in both species falls to one-half the peak values in roughly an hour (and this fall has been shown to be due to excretion) it may be concluded that excretion proceeds at a similar pace in the two species.

Compartmental distribution of borate. When a substance is injected into the body, there are 3 basic distribution patterns it may follow: 1) It may behave primarily as an extracellular constituent, like sodium, and distribute uniformly throughout the plasma, interstitial fluid and lymph—a volume representing some 20% of the body weight. 2) It may be distributed throughout the body water, both extracellular and intracellular, in essentially uniform concentration, in which case the diluting volume is about 65 to 70% of body weight. 3) It may reside primarily within the cells of the body, as is the case with potassium, and be concentrated many fold compared with the plasma level. In this case, the *apparent* diluting volume calculated on the basis of the plasma concentration may even exceed the body weight.

We have applied these principles to an analysis of the distribution data in nephrectomized mice in which we felt more confident that a true distribution equilibrium was reached. In Table III the data for the nephrectomized mice have been normalized to a standard dose of 10 γ boron per gram of body weight. From the averages of the equilibrium values, one notes that the concentration of boron in most of the organs, plasma and red cells is practically identical, and that the diluting volume is 100% of the body weight, suggesting the intracellular pattern of distribution (see No. 3, previous paragraph). Since plasma is about 92% water by weight, and the tissues about 75%, a uniform and exclusive distribution in body water would require that the tissue concentrations be about 82% that of the plasma. Actually they turn out to be 90-100%, and we have sought a particular reservoir for the additional 10-20% in specific abdominal organs, bone, and fat. None of these accounts for the excess, which must then represent an intracellular accumulation above that in general body water.

In the light of these conclusions it is reasonable to expect that the turnover rate of borate in its bound intracellular form will be different from the exchange rate of the freely diffusible ion. A more detailed graphic analysis of the blood data presented in Fig. 1B, supplemented with data extending out to 24 hours, shows in fact that its clearance is more accurately represented by 2 exponentials having the following formula:§ Excretion $= 88\, e^{\frac{.693}{60}t} + 6\, e^{\frac{.693}{330}t}$ where t is in minutes. From this equation we may deduce that if injection and mixing were instantaneous, the initial level would be 94 γ/g (the sum of the

§ In this formulation the denominator of each exponential is its half-time.

coefficients), representing a dilution volume some 35 to 40% of the body weight. Evidently, at the peak, borate is distributed beyond the extracellular space but incompletely in the intracellular space before excretion begins to play a substantial role. This equation indicates further that about 94% (88/94) of the boron in the body is excreted with a half-time of 60 minutes (compared with the earlier estimate of 65 minutes for the total clearance rate), and the remaining 6% with a half-time of 5.5 hours. Thus the faster of these represents the renal excretion rate of the freely diffusible borate, and the slower one would appear to be the turnover rate of the bound fraction.

These two phases of borate distribution also bear an important relationship to the proportionality between dosage and tissue levels. We have seen in the dosage study that during the early hours after injection (*e.g.,* during the predominantly aqueous phase of distribution) dosage and tissue levels are directly proportional over a wide range. However, with heavier doses this correlation broke down at 6.7 hours, since then the tissues all contained roughly 5 γ/g of boron regardless of whether the dose was 5 X or 20 X. Apparently, when the aqueous phase has been largely depleted by excretion, the role of the tissue-bound borate becomes relatively greater and affects the proportionality adversely.

Factors determining tumor-tissue ratios. We have seen that borate is distributed rapidly to tumor and the various organs *except brain,* and that high, useful concentrations and tumor-brain ratios occur during the first hour after injection, when rates of absorption and distribution are the controlling factors. Both concentrations and ratios fall precipitously after the first hour, however, due to the controlling effect of renal excretion. Although within the first hours after injection borate is distributed uniformly throughout much of the body, with the passage of time the normal brain retains a greater and greater percentage of that which remains. Thus the concentration in the brain in our nephrectomized mice was still rising at 4 hours; in one of our rabbits at 24 hours the borate in the brain had risen to 75% of that in the other tissues; whereas in Pfeiffer's dogs(11) at 7 days the brain contained a higher concentration than any other organ. This unusual predilection for nervous tissue, with its accompanying toxic effects, limits the frequency with which boron-neutron treatments may be given. Happily this relative affinity does not become manifest at once so that during the initial period of rapid exchange and high concentrations, when most of the boron is in the aqueous phase, the brain concentration is relatively low and the tumor-brain ratio of a useful magnitude. In other parts of the body where borate diffuses freely and there is little specific affinity among the organs or tumor, the concentration ratios are essentially unity. Unfortunately this state of affairs offers little immediate promise for the use of boron-neutron treatment for neoplasms other than those of the central nervous system.

Summary and conclusions. 1) Recent application of the atomic pile to the neutron-capture therapy of human brain tumors has focussed attention on the biological behavior of boron compounds. 2) Studies reported in mice and man indicate that borax and boric acid are rapidly absorbed and distributed throughout the body. Uptake by the various organs and tumor describes a family of similar curves such that the faster the rise in concentration, the higher and earlier is the peak. All tissues studied, except brain, reach a peak by 30 minutes. Borate is rapidly excreted by the kidneys as a "low-threshold substance" in a class with urea and sulphate, and clearance from the blood in mice proceeds with an exponential half-time of approximately 65 minutes. 3) Concentrations of borate in the tissues of normal mice are directly proportional to dosage for at least 2 hours after injection over a range of 18 to 700 μg/g body weight. 4) At equilibrium distribution, studied in nephrectomized mice, borate is uniformly distributed throughout the body water, with a 10 to 20% excess bound in the intracellular compartment. This excess is bound more tenaciously in brain than in other tissues and appears to be responsible for the toxicity of these compounds. 5) Study of the factors determining the concentration ratio between neoplasm and surrounding normal tissues indi-

cates that boron-neutron treatment offers little promise for malignant tumors other than those of the brain.

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Action of Hydrocortisone on Cells in Tissue Culture.* (21011)

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Since it was established that cortisone inhibits production of granulation tissue in experimentally produced wounds(10), much work has been conducted by numerous investigators studying the direct effect of cortisone on cells in tissue culture. The results have been diverse and in some instances have been contradictory. To mention only a few, inhibition of growth of fibroblasts by cortisone acetate in suspension in concentrations of about 200 to 50 μg/ml (reviewed in 4,2,6), and also by doses as small as 0.5 and 0.3 μg/ml(1,4) has been reported. On the other hand, negative results have been obtained with doses as high as 500 μg/ml (reviewed in 4,8). Stimulation of growth of fibroblasts(6), but also inhibition(8) by desoxycorticosterone have been reported.

In view of the above, attempts have been made to ascertain whether an easily reproducible effect of cortisone on growth in tissue culture, if any, could be demonstrated.

Methods. Primary cultures were used since such growth seemed to be more comparable to growth in regenerating wounds than would be the case if older laboratory strains were used which in long generations may have acquired different qualities. When the first experiments appeared inconclusive, it was thought results might be more clearcut, if embryonic extract (EE) was omitted from the culture medium. It appeared unreasonable to add so potent a growth stimulator as EE, to compete with the substance to be tested, particularly since the active principle of EE is unknown. A medium of amniotic fluid alone, without EE, has been found very satisfactory for obtaining adequate growth in control cultures. In all experiments the hanging-drop method on coverslips was used. In one series of experiments cultures of chick embryo tissue were explanted on coverslips in

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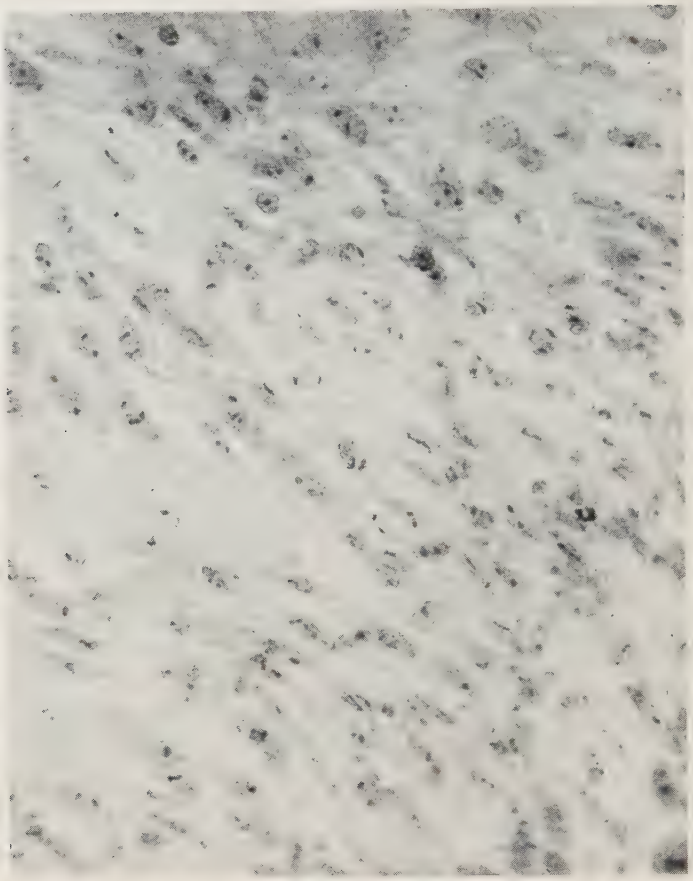

FIG. 1. Fibroblasts from chick embryo heart grown in chick plasma and amniotic fluid. Magn. 350 ×.

a liquid medium without plasma, the medium consisting of equal parts of chick amniotic fluid(3) and chick EE. Growth was obtained in about 70% of cultures and in general was not inferior to growth in a plasma clot. For all other experiments a plasma clot medium was used, to which either chick amniotic fluid plus 30% chick EE, or chick amniotic fluid alone, was added. In most experiments tissue fragments from hearts of 10- to 12-day-old chick embryos were used for explantation; chick embryo stomach and intestine were also used. The substance to be tested was added to the medium at the time of explantation. Whenever possible, water soluble compounds of steroids were employed since suspensions may be inhomogeneously distributed in individual cultures. In most experiments, the free alcohol of hydrocortisone† (17-hydroxycorticosterone, compound F, hereafter referred to as F alc.) was tested. To one ml containing 25 mg F alc., after removing the original vehicle (0.9% benzyl alcohol) and replacing it by saline, 1.25 cc 95% ethyl alcohol and 0.75 cc distilled water were added in the order mentioned. A clear and stable solution containing 8.33 mg F alc. per ml was obtained; this was diluted in 9 parts of Ringer solution. The final solution for testing in tissue culture was made with chick amniotic fluid. When the vehicle for F alc. was diluted in a similar fashion, no effect on cultures was noted. Four hundred and sixty preparations were made in studying F alc. in solution; F alc. as a suspension was employed in 80; cortisone acetate† in suspension in 80; desoxy-

† Kindly supplied to us by Dr. Elmer Alpert of Merck & Co., Rahway, N. J.

FIG. 2. Fibroblasts from chick embryo heart grown in chick plasma and amniotic fluid containing 250 μg/ml soluble hydrocortisone. Magn. 350 ×.

cortisone glucoside in suspension‡ in 40; and in aqueous solution§ in 40; and cholesterol in suspension† in 80. A smaller number of controls was run with each experimental set. Estimations of growth rate were made daily for 4 days. Area was estimated by recording the number of visual fields at a given microscopic magnification occupied by outgrowth, starting from the edge of the explant. Two zones in each culture were recorded, the largest and the smallest. Estimation of density was recorded by numbers 0 to 4. The average outgrowth area and density of a set of cultures gave a number characteristic for the medium used. Such results appear to agree with those obtained by measuring projectoscopic drawings with a planimeter, insofar as area is concerned; more emphasis, however, has been put on density. The described method is satisfactory for establishing large effects on growth in tissue culture, but is not sufficient for finding small differences which do not exceed considerably the fluctuations of normal cultures.

Results. At first, a liquid medium consisting of equal parts of chick amniotic fluid and chick EE was used with soluble F alc. in a final concentration of 250 μg/ml. Reproducible growth inhibition occurred in this medium containing soluble F alc. Growth of control cultures underwent such large fluctuations, however, that use of a liquid medium was abandoned and a medium containing plasma used. With chick plasma containing 20 to

‡ Kindly supplied to us by Dr. Yonkman of Ciba Pharmaceutical Co., Summit, N. J.

§ Kindly supplied to us by Dr. Kenneth Thompson of Roche-Organon, Nutley, N. J.

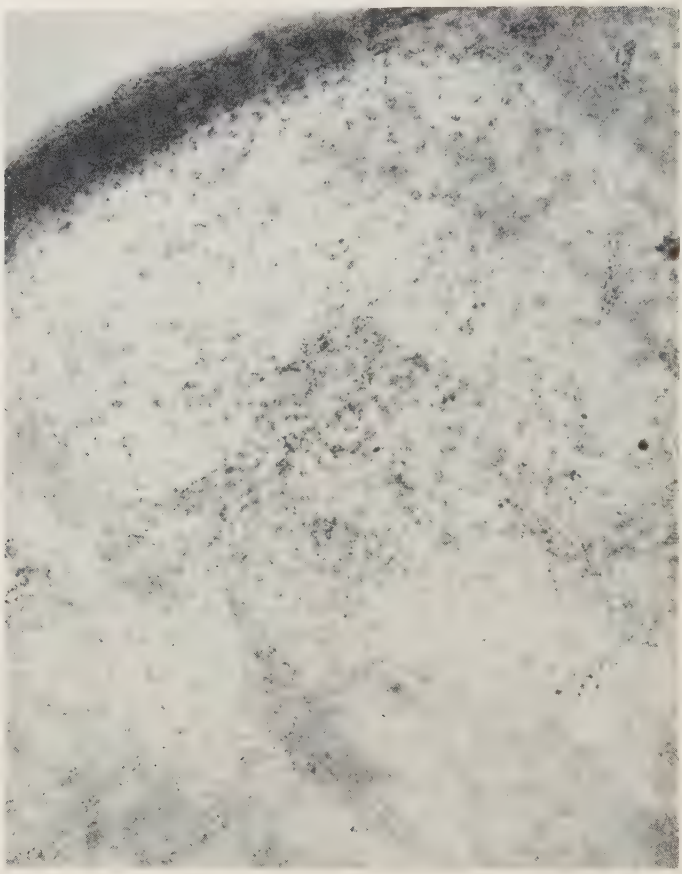

FIG. 3. Epithelium from chick embryo stomach mucosa grown in plasma and amniotic fluid containing 250 μg/ml soluble hydrocortisone. Magn. 150 ×.

50% of EE in amniotic fluid, the effect of F alc. varied greatly from experiment to experiment and often no effect was seen. In media containing equal parts of plasma and amniotic fluid without EE, with addition of 200 to 400 μg/ml of soluble F alc. in final concentration, the results were clearcut and easily reproducible. The difference in growth area and density between F alc.-treated and control cultures was large and the growth inhibition considerable. Higher concentrations of 400 to 500 μg/ml inhibited growth completely or almost completely. Concentrations of 100 and 50 μg/ml often showed slight inhibition which, however, was not always reproducible with this method.

Mitotic figures were encountered, but more rarely than in control cultures. Though mitotic indices were not counted at this time, no arrest of mitosis in a particular phase could be noted and there was no accumulation of one type of mitotic figure at any day of cultivation.

The presence of large fat vacuoles in an unusually early stage of growth was noted in cultures submitted to the action of soluble F alc. These have been found by others (9,11) and have also been seen in cells in tissue sections(1,9).

When cultures, treated with soluble F alc., 250 to 400 μg/ml, showing typical growth inhibition, were transferred on the 3rd or 4th day of cultivation into a medium of amniotic fluid without F alc., complete recovery of the cultures and excellent growth equal to that of control cultures ensued.

Aqueous suspension of cortisone acetate tested in a medium of plasma and amniotic fluid in the same concentrations as soluble F alc. caused slight inhibition of growth, con-

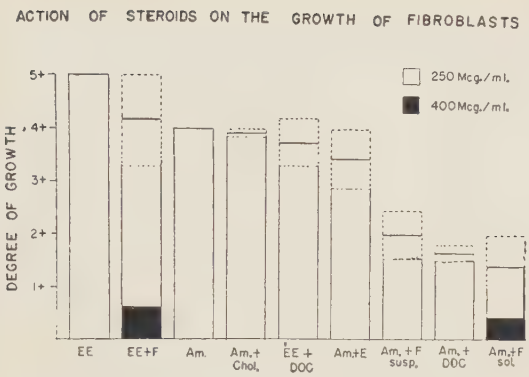

FIG. 4.

Straight lines at top of each column represent avg degree of growth, and fluctuations in growth are represented by broken lines. EE—embryo extract, F—compound F alcohol, AM—amniotic fluid, DOC—desoxycorticosterone glucoside, E—compound E.

siderably smaller than the inhibition by soluble F alc. Inhibition by F alcohol in suspension was greater than by cortisone acetate in suspension indicating also that factors other than water solubility make compound F more effective than cortisone acetate.

Two preparations of desoxycorticosterone glucoside (DOC), one in aqueous suspension and one in aqueous solution, were tested in the same medium and concentrations as above. The extent of growth inhibition caused by these steroids was quite similar to that obtained when soluble F alc. was used. It would thus appear that, as far as the action on fibroblast growth is concerned, DOC is not antagonistic to cortisone, but exerts a strong inhibitory effect similar to that of soluble F alc. Differences in cell morphology between DOC-treated cells and F alc.-treated were noted which are now under study. Cholesterol in suspension produced a slight degree of growth inhibition in tissue culture. Most of the experiments were carried out with soluble F alc. The inhibition produced by this compound was typical and reproducible. Hyaluronidase did not interfere with the F alc. inhibition.

Addition of various amounts of EE to a medium containing soluble F alc. in concentrations of 200 to 250 μg/ml, as mentioned above, gave better growth than a medium with soluble F alc. in the same concentrations without EE. (In most instances, the difference in growth between soluble F alc.-treated cultures with and without EE was greater than the differences in growth between control cultures with and without EE. The F alc. inhibition appeared thus to be absolutely smaller in the presence of EE. This would imply that EE partly removes the inhibitory effect of F alc., and suggests that EE may contain some principle antagonistic to the F alc. inhibition.) It should be emphasized, however, that the action of F alc. with EE depends on relative concentrations of both. When the concentration of soluble F alc. was raised to 400 μg/ml, inhibition was as great in the presence of EE as in amniotic fluid only.

Experiments with explants of stomach and intestinal epithelium were carried out in 2 media: first, one containing soluble F alc., 250 μg/ml amniotic fluid without EE, and second, in a medium of 400 μg/ml of soluble F alc. per ml amniotic fluid containing in addition 15% EE. Control cultures were grown in the same media without F alc. In the F alc.-treated cultures, growth of fibroblasts was considerably inhibited, whereas growth of epithelium in large sheets was abundant. In the control cultures, fibroblasts grew as usually, often abundantly, where the medium was not liquefied. (Stomach epithelium may cause liquefaction of the plasma clot. Under these circumstances, fibroblasts grew poorly and these cultures were discarded.) Big sheets of epithelium also grew irregularly in control cultures.

The same results were obtained when, in the same clot containing soluble F alc. in amniotic fluid, 2 tissue fragments were explanted, one from chick heart, and one from stomach or intestine. Controls were made with similar pairs of explants without F alc. Suppression of growth of fibroblasts in heart as well as in explants from stomach and intestine, and growth of large epithelial sheets in explants from stomach and intestine were noted. In control cultures there was normal growth of fibroblasts from all explants, and less vigorous growth of epithelium from stomach and intestine explants. It seems probable that F alc. could be practically used for suppressing growth of fibroblasts and indirectly enhancing

growth of epithelium in tissue culture.

Discussion. The first question suggested is whether the growth inhibition effected by F alc. in tissue culture is related to known effects of cortisone *in vivo, e.g.,* retarding granulation in experimentally produced wounds in animals. At first glance, the effect of a water soluble steroid—F alcohol—appears to mimic *in vitro* its accepted *in vivo* action. Chick embryo growth *in vivo* is inhibited by 11-oxysteroids; compound F acetate is the most potent(5). The selectivity of the growth inhibition on growth of fibroblasts with an absence of growth inhibition of stomach and intestine epithelium correlated with *in vivo* experience. Reversibility of inhibition when the steroid is removed is also compatible with *in vivo* findings.

On the other hand, DOC (granted in relatively high concentration) exerted an effect similar to F alc. on growth inhibition; even cholesterol apparently effected a minimal inhibition. This, particularly the DOC experiments, is contrary to *in vivo* work. The effect, however, is exerted upon the fibroblast and not upon the epithelial cell. The role of EE in this pattern is not completely clear. It does seem to negate partly the effect of compound F but the reason for its interference with the action of compound F has not been elucidated.

Summary. 1. In a medium consisting of chicken plasma and amniotic fluid, growth inhibition by soluble F alc. in concentrations from 200 μg/ml can be demonstrated consistently. 2. When EE is added to the culture medium, growth inhibition occurs only in a higher concentration of soluble F alc. The suggestion that EE contains a principle antagonistic to the inhibitory action of F alc. is discussed. 3. Growth of epithelium from stomach and intestine is not affected by F alc., while growth of fibroblasts is suppressed. 4. Reversibility of the effect of F alc. after its removal from the medium, and its selective inhibitory action on fibroblasts indicate that its inhibitory action *in vitro* is related to its known action *in vivo,* in particular to the retardation of regeneration in wounds experimentally produced in animals. 5. Aqueous soluble DOC in similar concentrations has an effect similar to soluble F alc. and cholesterol in suspension has a minimal inhibitory effect on cell growth. This observation differs from the *in vivo* findings and warrants further investigation.

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An Upper Limit for Acetylcholine Content and Synthesis in Human Erythrocytes.* (21012)

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This study was made as a result of a revival of interest in the possibility of acetylcholine† (ACh) synthesis in mammalian red cells. Physostigmine disturbs the electrolyte transport process in erythrocytes and the poor stability of canine cells in isotonic KCl is improved by adding ACh(1-3). It is well-known that the synthesis and breakdown of the ester are intimately linked with excitability in several tissues and thus inferentially with cation movements(4,5), but the basic mechanism of the maintenance of stable concentrations of cations in red cells remains a matter of speculation. If ACh metabolism is related to cation movements in the red cell, it should be possible to show that the order of magnitude of ACh turnover is large enough to furnish the minimum free energy required for the accumulation of K ion against a concentration gradient. The order of magnitude of ACh turnover may be estimated from the data presented in this communication.

Methods. Blood was drawn in the laboratory from several apparently healthy donors.‡ The samples were either lightly heparinized or defibrinated immediately, and spun in the cold. In some experiments, physostigmine was added to the blood immediately after it was drawn, with no demonstrable effect on the ACh content. Samples were usually taken in plasma or serum but washing the cells with saline did not appear to alter the results. Hemolyzates were prepared by rapid freezing and thawing of the packed cells. Samples of whole blood or cells in saline were usually 10 ml in volume and were precipitated with 5 ml of 20% TCA, yielding approximately 10 ml of extract of cell-saline suspensions or 6 ml of whole blood extract. TCA was removed by ether extraction. In some experiments, K was removed by ion exchange(6), the sample being poured through a column of IRC-50 ion-exchange resin, 1 cm in diameter and 2 cm long, initially in the Na form. Following this, elution was begun with 0.85% saline until a total of 30 ml had been collected. Since the bath volume on the muscle was 7.5 ml 4 samples were available for direct application to the test preparation and for the preparation of the usual blanks and calibration standards containing known amounts of added ACh. In testing for *choline acetylase activity*,§ red cells obtained from heparinized or defibrinated blood were treated with 10 volumes of analytical-grade acetone at –13°C for 20 minutes. This procedure was repeated and the suspension filtered, washed with acetone, and dried briefly under vacuum at 2°C. The product, a fine, brick-red powder, was stored over a desiccant at –20°C. Powders were used within 2 to 24 hours after preparation, with a few exceptions. Rat brain powders were prepared by a method similar to that suggested by Feldberg(7). Acetone-dried powders were suspended in ice-cold saline containing 0.17 g % L-cysteine. The usual concentrations of powders were: red cells, 80 mg/ml; rat brain, 15 mg/ml. Suspensions were homogenized in a Potter-Elvehjem homogenizer for 2 minutes at 15-minute intervals for 1 hour. The homogenates were spun and the supernatants were used without further treatment. Preparations were kept cold at all times. *Acetylation tests* were run in Thunberg tubes at 38°C for 1 hour. Incubates, usually 5.5 ml, contained 3 ml of

* Work performed under Contract for the Atomic Energy Commission.

† Abbreviations are as follows: ACh, acetylcholine; TCA, trichloroacetic acid; ATP, adenosinetriphosphate; CoA, coenzyme A; DFP, diisopropyl fluorophosphate.

‡ Thanks are due to various colleagues who generously donated blood for the various experiments, and to Dr. K. C. Atwood and Miss Blanche Gibbs who assisted in performing venipunctures.

§ Dr. Margaret E. Greig materially contributed to the work by generously sharing her technical experience with us.

extract and included in final concentration 6 mg of KCl, 3-6 mg of choline chloride, 4 mg of $MgCl_2 \cdot 6H_2O$, 15-30 mg of sodium citrate, 1.5-6.8 mg of sodium acetate, 2 mg of NaF, 5.4 mg of $Na_2HPO_4 \cdot 7H_2O$, 0.6 mg of physostigmine salicylate or 0.2-0.9 mg DFP, 4-8 mg of Pabst sodium ATP, usually both 0.4 mg of Pabst CoA and 0.3-0.4 ml of boiled yeast juice (200 mg of yeast/ml) and 0.85% NaCl to make the volume to 5.5 ml. In some experiments, either the CoA or the yeast was omitted. Normally, the ratio of K^+ to Na^+ was approximately ¼ but, in some experiments, K^+ was substituted for Na^+. Three experiments at an approximate K^+/Na^+ ratio of 4, one at 1/1 and 3 at ¾ were conducted. Syntheses were conducted under an atmosphere of 95% N_2-5% CO_2 and, at their conclusion, the incubates were inactivated with TCA or HCl, and centrifuged. In some cases, TCA was removed with ether but in others samples were neutralized with NaOH since only small amounts of protein precipitant were required. Various control syntheses included incubation with ACh to determine any systematic loss, and incubation in the absence of any one or a combination of the following: choline, acetate, citrate, CoA, physostigmine. Yields are reported as the amount in micrograms synthesized during one hour of incubation per gram of dry powder (γ/g/hour). The *physostigmine-sensitized* frog rectus abdominis used by Chang and Gaddum(8) and the acetone-treated rectus abdominis method suggested by Chang *et al.*(9) were used for the biological estimation of ACh. Our best results‖ were obtained with *Rana pipiens* and, on the whole, results with *R. clamitans* were inferior. Frogs were kept successfully for considerable periods in aquariums containing about 1 inch of water at 10°C. Large, egg-carrying females often seemed particularly satisfactory. Contractions of the rectus abdominis were recorded electrically on a strip paper chart(10). When physostigmine potentiation was to be used, the dissected muscles were stored in amphibian Locke solution in the cold for about 18 hours prior to assay, with generally improved results. On several occasions, muscles retained adequate sensitivity for as much as 5 days. Estimations in the range of 0.15-0.5 γ of ACh in a 7.5-ml bath were made by this method. For assays in which the acetone-treated rectus was used, muscles were dissected immediately before use. Sensitivities were in the range of 0.025-0.25 γ in a 7.5-ml bath containing 0.05-0.2 ml of acetone.

Results. The presence of ACh in TCA extracts of treated and untreated cells could not be demonstrated by the physostigmine-sensitized rectus. Cells were incubated at 25° and 38°C for 1 to 9 hours in the presence and in the absence of the cholinesterase inhibitors physostigmine, 1×10^{-4} to 8×10^{-3} M, and DFP, 5×10^{-6} to 1×10^{-3} M. Esterase-blocked cells were equilibrated in their own plasma and in suspensions of saline with and without added dextrose. Accumulation of ACh in suspensions of cholinesterase-blocked cells and hemolyzates at physiological temperature could not be detected aerobically or anaerobically. The addition of choline, acetate, and citrate did not alter the result. In some cases, ATP-ase was blocked with fluoride to preserve the normal ATP of the cells. Hemolyzates incubated anaerobically with the complete synthetic system used in the tests with acetone powder extracts synthesized less than 0.3 γ of ACh per ml of hemolyzate. The limit of detection in the remainder of the experiments varied between 0.26 and 0.09 γ of ACh per ml of packed cells. ACh was not detected in this range in any of the experiments. The complete recovery of added ACh in quantities of 0.1-0.4 γ per ml of cells assured no systematic loss.

Additional experiments in which slightly higher sensitivity of detection was achieved by acetone potentiation indicated that red cells contain less than 0.08 γ of ACh per ml. No detectable increase could be observed by incubation for 2 hours in the presence of esterase inhibitor. Small contractions occurred in some instances, but they were either unaltered or were increased by alkaline hydrolysis. They were thus not caused by ACh.

The estimated upper limit of ACh synthesis

‖ The authors wish to acknowledge the friendly cooperation and advice of Dr. S. R. Tipton and Dr. Karl Wilbur.

by acetone powders of red cells was compared with syntheses by rat brain controls. It is clear from the discussion of *methods* that syntheses were conducted under a variety of experimental conditions, *i.e.*, the age of the red cell powders (prepared from the blood of several donors) was varied; either DFP or physostigmine was used to block cholinesterase; either CoA or yeast juice was frequently omitted; the ATP concentration was varied; either acetate or citrate or both were used as substrates. The results were unaffected by any of these modifications with the exception of the last. Brain extracts synthesized between 430 and 1040 γ ACh/g/hr if either acetate or citrate was used as the substrate and under these conditions synthesis by red cell powders was below the threshold of detection (2.8-13.2 γ ACh/g/hr). The inclusion of brain activator(11) in one experiment did not affect the result. Small contractions were occasionally observed at this level but they were shown to be artifacts. Results of additional experiments showed that maximal brain syntheses (1200-1670 γ ACh/g/hr) were obtained if *both* acetate and citrate were used as substrates. Under these optimal conditions small amounts of biologically active material were frequently produced by extracts from red cell powders (1.1 or less to 6.3 or less γ ACh/g/hr). Inclusion of purified pigeon liver extract did not materially improve the synthesis(12). Extracts from a system in which the white cells had been concentrated showed no increased synthesis. Red cell synthesis was not reduced if the *dry* powder was heated in a boiling water bath for 1 hour, or if the powder was extracted without cysteine and the extract incubated with $CuSO_4$. Synthesis by blood-brain extracts treated similarly was reduced by 50%. Synthesis by brain alone was reduced by 80% if the powder was extracted without cysteine and the extract incubated with $CuSO_4$. In addition, synthesis by red cells was not altered if the *extract* was heated at boiling temperature for a short time but synthesis was markedly reduced if boiled extracts were redried with acetone, the powder obtained extracted with saline, and incubated as a typical extract. The effect of the synthesized material was reduced by boiling at alkaline pH but was not enhanced by treating the rectus with physostigmine, whereas extracts containing added ACh showed physostigmine potentiation. It was found that the activity was unaltered by atropine. No increase in biological activity was produced in syntheses at high K^+/Na^+ ratios. Syntheses were clearly less than 3 γ/g hour. Alkaline hydrolysis generally produced less reduction of the small biological effect, which was entirely the same with or without physostigmine treatment. Recovery of ACh added in amounts which correspond to synthetic rates of 3-100 γ/g/hr was quantitative in the experiments reported.

TABLE I. Effect of Red Cell Powder on the Rate of ACh Synthesis by Brain Preparations. Brain powder extract 30 mg/ml, brain-blood powder extract 60 mg/ml concentrations in saline-cysteine solutions. Amount of extract shown as equivalent mg of powder extracted. Synthesis in γ/g/hr.

Brain		Brain-blood		
		Amount (mg)		
Amount (mg)	ACh synthesis (γ/g/hr)	Brain	Blood	ACh synthesis (γ/g/hr)
97.3	1600	100.3	100.3	760
48.6	1300	49.4	148.2	770
24.3	1200	22.5	157.5	1600

Inhibition of brain extract activity occurred in several experiments in which red cell powders were added during extraction. This was not invariable, however, and in one series of experiments (Table I) where particular care was used to maintain uniformity of extraction procedure, the largest ratio of blood powder to brain powder during the extraction actually produced a greater synthesis than in the control. Synthesis by brain extract was not affected by a hemolyzed red cell preparation.

Acetone effects on the muscle. Results of a number of experiments indicated that contraction of the rectus in the presence of acetone may constitute a less specific test for ACh than that provided by the physostigmine-sensitized preparation. Investigation of some of the properties of acetone potentiation included determinations of its effect on the choline, K, ATP, and ACh response of the untreated, physostigmine-sensitized, and atropinized rectus.

We found that, in the presence of ace-

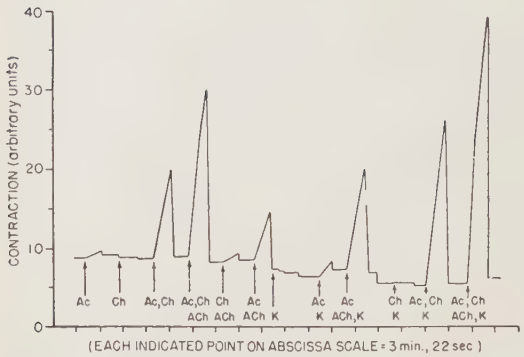

FIG. 1. The effect of choline, K, and ACh on the acetone-treated rectus. Each contraction recorded for 3 min. beginning at arrow (↑). Recorder off during 10-min. wash. Total bath volume, 7.5 ml including the following additions as noted in the figure: Ac = 0.1 ml of acetone, ACh = 0.1 γ of acetylcholine, Ch = 0.3 mg of choline chloride, K = 3 mg of KCl.

tone, the rectus was sensitized to choline and K as well as to ACh. Choline and K contractions resemble ACh contractions both in the shape of the curves described and in the rapidity of relaxation on removal of the stimulating agent. In addition, as with physostigmine potentiation, the ACh response of the acetone-treated rectus may be potentiated by choline and K in threshold and subthreshold concentrations. Fig. 1 illustrates some of the contractions which were recorded. In Fig. 2 it may be seen that physostigmine appears to *enhance* the effect of ACh on the acetone-treated rectus (contractions 1, 3, 5) and that atropine appears to counteract the effect of the physostigmine to a greater extent in the absence than in the presence of acetone (contractions 5 and 6, and 3 and 7), but that the *direct* action of ACh on the acetone-sensitized muscle is not depressed by atropine (contractions 7 and 1). It seems, then, that the acetone-treated rectus is subject to a number of effects which may simulate and/or potentiate the effects of ACh. The frog rectus may not serve as an adequate assay material in syntheses in which acetone powders of low activity are used without removing any acetone which the samples may contain. Threshold activity detected in these systems may be due to potentiation by residual acetone to ACh, choline, K, some other active substance, or to a combination of these substances. Loss of activity of the samples boiled at alkaline pH may mimic alkaline hydrolysis but may be caused by loss of acetone. The nearly equal response of the rectus to these samples in the presence or absence of physostigmine and atropine may be attributed to the presence of acetone. It is recognized that acetone potentiation can only partially account for the effects observed in our red cell experiments and that the results were probably also influenced by the presence of other unidentified substances.

Discussion. A number of reports in the literature attest to the low level of ACh in mammalian erythrocytes. Chang and Gaddum(8) showed that the level in dog, horse, and ox cells was 0.05-0.08 γ per ml of blood. The results suggested substances other than ACh may be responsible for biological activity detected at this level. Bülbring *et al.*(13) were unable to find ACh in rat cells. Synthesis by packed physostigmine-treated cells was undetectable. Quastel *et al.*(14) set the upper limit of synthesis in physostigmine-treated guinea pig blood at 0.3 γ per hour per 100 mg dry weight. Our results in man fully confirm

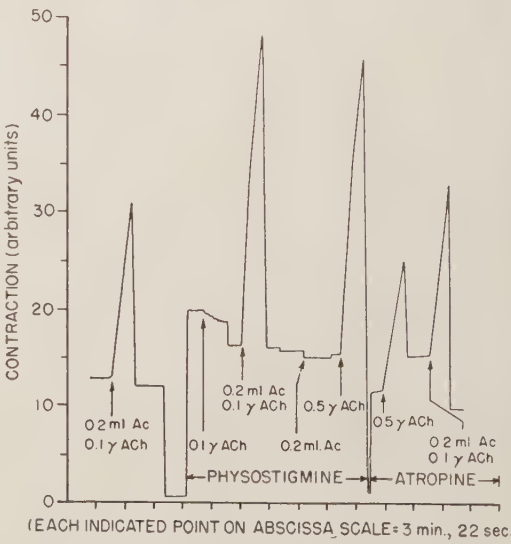

FIG. 2. The effect of acetone (Ac) on the ACh response of the untreated, physostigmine-sensitized, and atropinized rectus. The muscle was treated with 8×10^{-6} g/ml of physostigmine salicylate for 52 min. and atropinized with 6×10^{-7} g/ml of atropine sulfate for 20 min. Contractions recorded as in Fig. 1, bath volume as in Fig. 1. All additions to bath noted in figure.

the low level of erythrocyte ACh, and it would seem not unlikely that this will be generally true in mammalian red cells. The low value of synthesis is in accord with the low value of the observed choline acetylase activity but it could also be explained by postulating that there is at all times a small amount of bound ester which can be replaced by synthesis only when it is depleted. A simple calculation will show that if this were so and if all this material were uniformly spread on the surface of the erythrocyte each molecule would occupy approximately 5×10^{-3} square micra with a spacing of at least 700 Å, or approximately one percent of the diameter of the erythrocyte. Of course the possibility remains that ACh exists with closer spacing in only a portion of the cells.

The observed synthetic power of the cell powders is less than that which has been previously reported(15) and is exceedingly low compared to that found in tissues in which ACh is known to play a physiological role. When choline acetylase activity from an outside source is present most and sometimes all of it can be observed in red cell powders. Bülbring *et al.*(13) were able to observe synthesis of ACh by trypanosomes in the presence of red cell material. Although some inhibition by red cell powders is occasionally observed in our experiments it is never more than 50%. Even in the worst cases there is enough synthesis by brain enzyme in the presence of red cell material to indicate that inhibition alone can scarcely account for the low level of synthesis by red cells. Since red cell synthesis is not affected by treatment which reduces brain synthesis by 80%, it is possible that the activity observed is not the result of synthesis by choline acetylase. Nonenzymatic synthesis or synthesis by some other enzyme may occur. It is possible, of course, that choline acetylase activity exists in red cells but, in contrast to neural tissue, cannot be demonstrated by the standard procedures of extraction. A more likely interpretation is that the low level of synthesis reflects a general low level of acetylcholine metabolism in erythrocytes whose generally low metabolic activity is familiar.

The effects of physostigmine and DFP on ion transport in erythrocytes(1,2) suggest some relation between cholinesterase activity and ion movement. If the turnover of ACh could be verified and related in some way to the process of selective K accumulation in red cells, then the energy required could be conveniently related to the energy released in the hydrolysis of ACh. If the observed upper limit of choline acetylase activity in our experiments is accepted, the result is not encouraging for an ACh mechanism. Current estimates of the minimum free energy required to accumulate a K ion against the concentration gradient in human cells are about 1600 calories/mole(16). The hydrolysis of 1 mole of ACh yields not more than 3200 calories (17). Thus not more than two K ions can be accumulated per mole of ACh hydrolyzed if such a process is to account for the energy of selective K accumulation. Since about 2×10^{-6} equivalents of K exchange per hour in 1 ml of human cells(18) at least 10^{-6} moles of ACh would be synthesized per ml of cells or about 150 γ of ACh per ml. Since the ratio of the weight of packed cells to that of the acetone dried powder is about 3:1, this is at least 50 times as great as any synthesis which we have observed from powder extracts. Certainly synthesis of 3 γ/g/hr in physostigmine treated cells would have been detected. The result suggests that, even with a liberal allowance for inferior synthesis *in vitro* and other factors, ACh turnover can scarcely be involved in selective K accumulation in erythrocytes. This conclusion does not deny the possibility that cholinesterase in the envelope might play some role other than that involving the hydrolysis of ACh, but this remains to be elucidated.

Summary. The ACh content of human red cells is less than 0.08 γ/ml and no increase in synthesis can be observed in cells equilibrated with cholinesterase inhibitors. A synthetic rate higher than 6.3 γ/g/hr by acetone powders of red cells was not observed. There is some doubt that the activity observed was the result of enzymatic synthesis of ACh. Calculations show this rate to be less than 1/50 that required to provide the free energy essential for the accumulation of K against a concentration gradient. The metabolism of

red cell ACh as a mechanism by which the cells accumulate K is of doubtful importance.

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Chylomicron and Clearing Reaction: Effect of Heat and of Refrigeration.* (21013)

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The *in vitro* reaction by which turbid fat suspensions may be cleared following the addition of plasma obtained from animals after the injection of heparin has been studied extensively by Anderson and Fawcett(1), Anfinsen *et al.*(2), and French *et al.*(3). The present study is concerned with the fat particles (chylomicra) themselves and with factors which alter their susceptibility to clearing.

Method. Samples of lipemic plasma were obtained from dogs approximately 3 hours after fat meals of 4-8 g/kg. Cream fat was fed except where indicated. The plasma samples were centrifuged (at 4°C for 30 minutes at 20,000 × G.) in a Servall angle centrifuge and the subnatant clear plasma removed. This was replaced by 0.85% NaCl, and the suspension was recentrifuged. This operation was repeated 3 times. The resulting saline suspension of washed chylomicra was used at once or stored at 4°C or −18°C and used within a few days. Chyle was obtained by cannulation of the thoracic duct of dogs 1-2 hours after feedings of 8 g of cream fat per kg, and washed suspensions of chylomicra from the chyle were prepared as described for the plasma. All clearing tests were performed as follows: 1 ml of chylomicron suspension (or lipemic plasma or diluted chyle) plus 4 ml 0.85% NaCl were warmed at 37°C for 10 minutes in a 19 mm Coleman cuvette. One ml of warm post-heparin plasma was then added, the time noted and the turbidity of the suspension determined immediately and at 10-minute intervals for one hour or longer. All determinations of optical density were made in a Coleman spectrophotometer at 650 mμ wave length. The tubes were incubated at 37°C in a water bath during the test. The samples of post-heparin plasma were obtained

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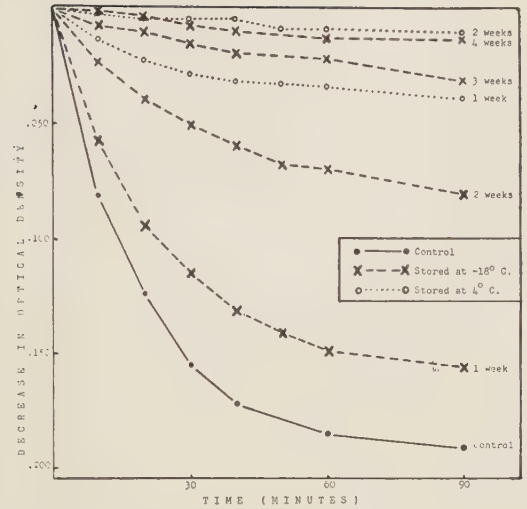

FIG. 1. Clearing factor tests using saline-washed chylomicra which had been stored at –18°C or 4°C for the periods of time indicated.

from dogs 10 minutes after the injection of 25-50 units of heparin (Connaught Laboratories) per kg body weight. These were stored at –18°C until used. We found no decrease in potency of these samples after storage for one month at this temperature. In all cases blood samples were drawn into 1/10 volume 3.8% sodium citrate solution. Chyle was also collected in the same anticoagulant. For the stability experiments 1.0 ml amounts of the chylomicron suspension to be tested were pipetted into tubes and subjected to different temperatures. The entire contents of these tubes were then used for the clearing test. For any one experiment aliquots of the same post-heparin plasma sample were used as the source of "clearing factor."

Results. Effects of refrigerator storage and of freezing. The susceptibility of washed chylomicra or of whole lipemic plasma to clearing by post-heparin plasma decreased both in rate and extent during storage at 4°C or –18°C. This change was marked at the end of one week and increased progressively with the time of storage (Fig. 1). Repeated (3 times) rapid freezing (to –18°C) and thawing (to room temperature) of washed chylomicra during a period of 5 hours had little effect on the susceptibility of the chylomicra to clearing.

Effect of heat. When washed chylomicra were heated to various temperatures no sharp inactivation temperature was observed; a gradual reduction in the susceptibility of different aliquots to clearing occurred as the temperature increased (Fig. 2). A gradual decrease in the susceptibility to clearing also occurred as the period of heating was increased (Fig. 3). Heating at 37°C for 15 minutes always caused a slight decrease in the susceptibility to clearing; a complete loss of the ability to be cleared was present after washed chylomicra had been heated for 3 hours at 100°C, or for 24 hours at 37°C.

Nature of fat in the chylomicra. In one experiment each of 3 dogs (siblings) was fed a different fat: cod liver oil in skim milk (4 g fat/kg body weight), glyceryl trioleate in skim milk (4 g fat/kg body weight) and 35% cream (6 g fat/kg body weight). The chylomicra obtained from each dog were washed 3 times in saline as usual. The samples were adjusted to exactly the same optical density, and aliquots of each were heated at 65°C for 30 minutes. Fig. 4 shows that the chylomicra from the dog fed glyceryl trioleate cleared more rapidly than the chylomicra from the cream-fed dog. The susceptibility to clearing of both suspensions was lessened to about the same degree by heating. Chylomicra from the

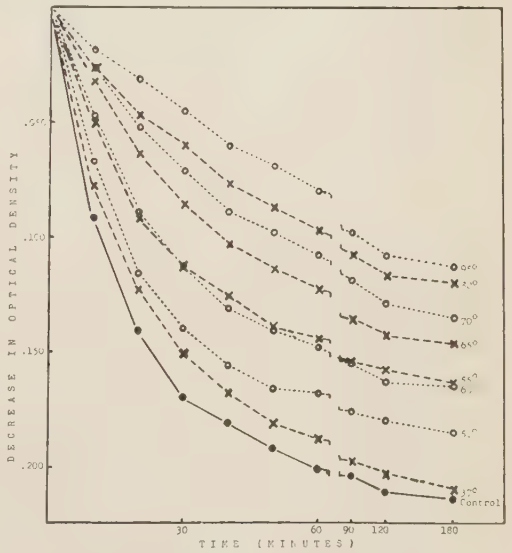

FIG. 2. Clearing factor tests (at 37°C) using saline-washed chylomicra which had previously been heated for 15 min. at the temperatures indicated.

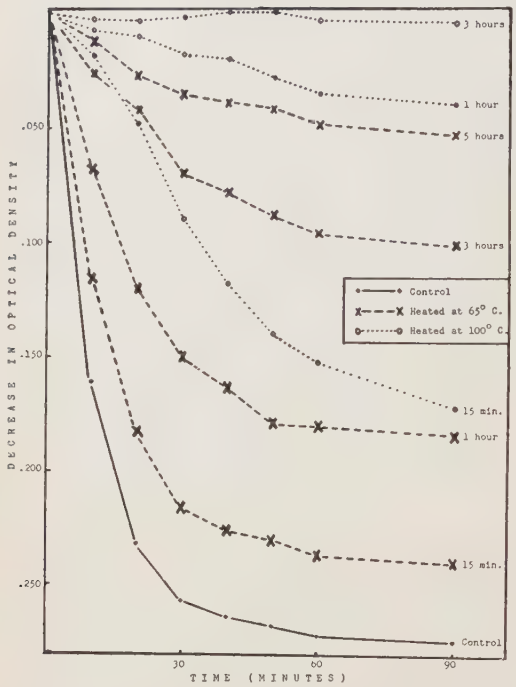

FIG. 3. Clearing factor tests (at 37°C) using saline-weshed chylomicra which had previously been heated at 65°C or 100°C for the periods of time indicated.

cold liver oil-fed dog cleared much less rapidly than did the chylomicra from the other 2 dogs, and after heating to 65°C for 30 minutes these chylomicra lost almost completely their ability to be cleared. Microscopic examination (dark field) of the unheated preparations showed that the "cod liver oil chylomicra" were much larger than the others. It is of interest that *in vivo* cod liver oil lipemias clear less rapidly following the injection of heparin than do lipemias following the ingestion of cream or glyceryl trioleate(4).

Experiments with added plasma. Many attempts were made to determine if the chylomicra which had suffered some loss of their susceptibility to clearing during heating could regain this property in the presence of fresh plasma. Various conditions such as the addition of different amounts of plasma with or without incubation for different periods of time, and centrifuging, washing and re-isolation of the chylomicra after the addition of plasma were tested. The results varied. In about half the experiments the addition of normal plasma to suspensions of chylomicra damaged by heating to 37°C or 65°C was attended by a partial recovery of the rate of clearing of the chylomicra. In no instance was the recovery complete. In a number of experiments 1.0 ml of normal plasma and saline in different proportions was added to 1.0 ml of washed, saline-suspended chylomicra. This was heated and then centrifuged to remove precipitated plasma proteins. The supernatant was tested for its clearing susceptibility. In all experiments the addition of 0.1 to 0.5 ml of plasma reduced the expected loss of susceptibility to clearing when heated to both 65°C and 37°C. The addition of 0.01 ml and 0.02 ml of plasma usually did not give this protection.

Experiments with chyle. Freshly obtained chyle, diluted approximately 1:100 with saline lost only a little of its susceptibility to clearing during heating at 50°C or 65°C for 15 minutes. Also when stored for as long as one month in the refrigerator or at −18°C in its

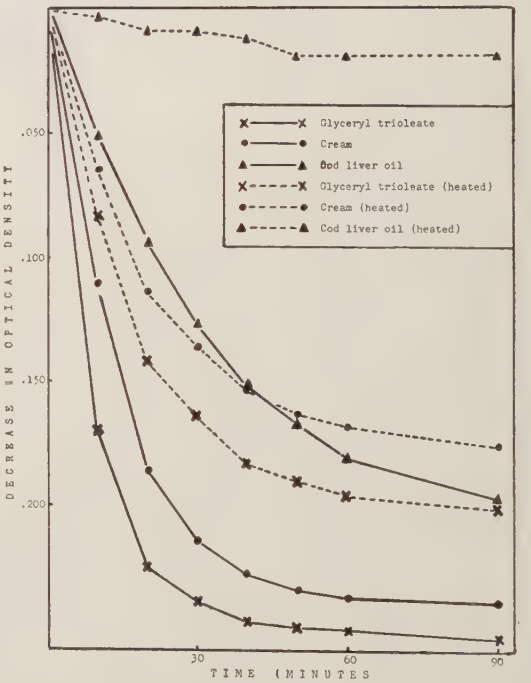

FIG. 4. Clearing factor tests using saline-washed chylomicra obtained from dogs which had been fed 3 different fats (as indicated). Broken lines show the same chylomicra tested after heating 30 min. at 65°C. All optical densities were the same at the beginning of the clearing test.

undiluted state, chyle lost none of its susceptibility to clearing. Saline-washed chylomicra from chyle, on the other hand, were significantly less susceptible to clearing after storage for one month at –18°C, although they suffered less in this respect than did saline-washed chylomicra from plasma. It was found necessary to warm the frozen chyle 2-3 hours at 37°C before testing, otherwise it continued to equilibrate during the testing and erroneously gave the appearance that its clearing susceptibility improved after heating at 50°C or 65°C. After the 3 hours prewarming, however, it was found that the previously frozen chyle showed the same degree of very slight "damage" after 15 minutes heating at 50°C or 65°C as the fresh chyle.

Discussion. Our results showed that the susceptibility of the chylomicra to clearing by post-heparin plasma may be reduced by storage at 4°C or –18°C or by heating. Whether this is due to changes in the fat of the chylomicron or in its protein envelop, or to changes in a lipoprotein complex cannot be concluded from our studies. If the changes are due to denaturation of a protein film on the chylomicron the question would still arise as to whether this film is essential in the sense that it is an essential factor for maximal clearing, or whether the denatured protein coating interferes with the clearing reaction by making the fat unavailable. It is of interest that loss of susceptibility to clearing brought about by heating the chylomicra was not completely reversed by the addition of normal plasma, but the presence of certain amounts of plasma during heating lessened this change.

The possibility must also be considered that a specific protein or lipoprotein complex is involved. This is suggested by the fact that the heating of chyle (diluted 1:100 in saline) caused much less change in the susceptibility to clearing of the chylomicra than the same heat applied to washed chylomicra from lipemic plasma. This might suggest that the protein moiety of the chylomicron is fundamentally important to the clearing reaction, or that chyle itself contains a specific substance necessary to maximal clearing that is either absent or present in relatively low concentration in plasma.

Conclusion. Heat or storage at –18°C is attended by a significant reduction of the susceptibility to clearing of saline washed chylomicra from plasma. Thoracic duct chyle is affected much less by the same treatments.

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Plasma Volume Changes in Egg-White Injected Rats. Effect of Cortisone.* (21014)

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Selye described an "anaphylactoid" condition produced by intraperitoneal injection of egg-white to rats(1) characterized by the development of edema. Further studies(2,3) have shown that adrenalectomized rats reacted to egg-white with a more severe condition, usually fatal. It was also observed that adrenal cortical extract(4) and cortisone (5) had a protective effect on the systemic actions of egg-white. The present report deals with plasma volume changes in egg-white-treated rats and attempts to explain the mechanism of the protective action of corti-

* These studies were aided by a contract between the Office of Naval Research, Department of the Navy and the University of Illinois.

sone in terms of these changes and their effect on capillary function.

Methods. Plasma volume was evaluated by means of the Evans blue method. Albino rats weighing between 135 and 150 g were anesthetized with Nembutal (30 mg/kg intraperitoneally). Evans blue was injected by way of the left jugular vein at the dose of 10 mg/kg. Thirty minutes after injection of the dye, the animals were bled by sectioning the blood vessels of the neck. Blood was collected into small beakers containing 0.6 ml of a 1.1% solution of sodium oxalate. The sample was immediately transferred to a Kolmer type sedimentation tube and centrifuged for 10 minutes at 2500 r.p.m. After taking a hematocrit reading (taking into account the volume of anticoagulant solution), the plasma-oxalate mixture was pipetted off and distilled water was added to reach a 1/20th dilution of plasma. The plasma dilution was read against a distilled water blank on a Coleman Model 14 spectrophotometer at 600 mμ. The readings were converted into μg of dye per ml of plasma by means of a standardization curve of Evans blue dissolved in distilled water. Plasma volume was calculated from the equation: $V_{ml/kg} = \frac{\text{dye injected } (\mu g/kg)}{\text{plasma dye } (\mu g/ml)}$ Plasma volume was also calculated from the hematocrit by equating the plasma ratio (P.R. = 100 — Hematocrit) with the plasma volume obtained by the dye method. In any given experimental group the plasma volume was calculated from the following equation: $V_{ml/kg} = \frac{(P.R.)_{exp}}{(P.R.)_C} \times V_C$; where $(P.R.)_{exp}$ is the plasma ratio of the given experimental group, $(P.R.)_C$ is the plasma ratio of the corresponding control group and V_C is the plasma volume of this control group as calculated from the dye concentration. No corrections were made for plasma trapping which can be considered negligible compared to the experimental changes. Microscopic observations were made on the rat meso-appendix using the method described by Chambers and Zweifach(6). The Krebs-Ringer formula(7) was used with addition of 3% gelatin for drip solution. Observations were made with the Spencer phase microscope at 100× and 200× for gross observations and 400× for a more detailed view of circulation in individual blood vessels. Adrenalectomies were performed under ether anesthesia and the adrenalectomized rats were maintained on 1% NaCl in their drinking water. All the animals were treated with egg-white prepared from pooled eggs, dialyzed overnight in the cold and lyophilized. For each experiment fresh solutions of the lyophilized material were made up in saline. Cortisone (Cortone acetate Merck) was given subcutaneously according to the following schedule: First injection 2 days prior to experiment, second injection the day before and third injection 2 hours before administration of egg-white. The dose indicated is the amount given in each injection.

Results. Plasma volume changes. In 8 normal rats the mean dye concentration was found to be 126.0 (±6.7) μg/ml of plasma, giving a mean plasma volume of 79.5 ml/kg of body weight. Hematocrit determinations in the same group of animals gave a mean plasma ratio of 51.7 (±1.1)%. It is legitimate to assume that this plasma ratio corresponds to a plasma volume of 79.5 ml and that any change in plasma volume, other factors being equal, will be reflected by a corresponding change in plasma/cell ratio. This assumption, although probably valid for the purposes of this study, calls for certain reservations which will be discussed below.

When groups of rats were injected intraperitoneally with 2 g/kg of egg-white, simultaneously with the development of edema, plasma volume changes were observed with both the dye and the hematocrit methods. Blood samples were taken from animals killed at varying intervals after the egg-white injection. The interval between dye injection and blood collection was 30 minutes all through the experiments. The results shown in Fig. 1 indicate that, on the whole, when dye concentration increases the plasma ratio falls; both indicating loss of plasma from the circulating blood. A discrepancy is noted, however, for the first half-hour when the dye concentration drops simultaneously with the plasma ratio. At the peak of the reaction (between 1½ and 2 hours) the plasma loss

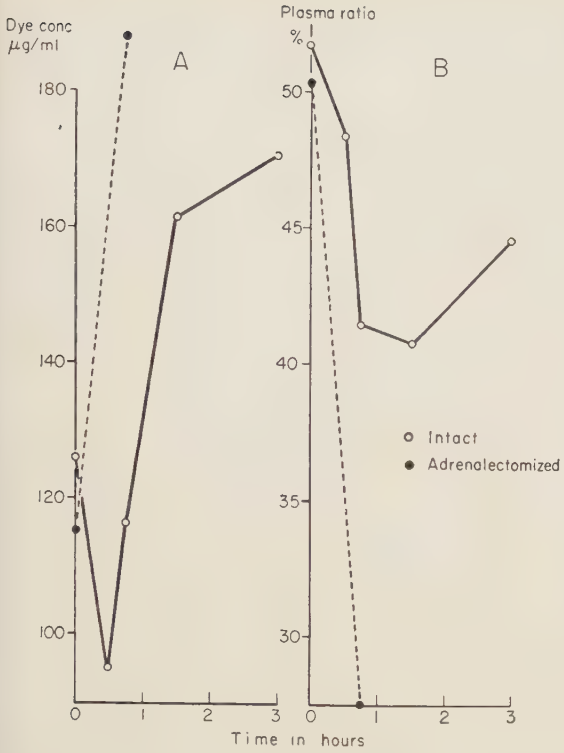

FIG. 1. Action of egg-white on plasma concentration of Evans blue and on plasma ratio in intact and adrenalectomized rats. Abscissa: time after intraperitoneal injection of 2 g/kg of egg-white (hr); ordinates, dye concentration in μg/ml of plasma (A), plasma ratio (= 100 − hematocrit) % (B). The rats were injected with 10 mg/kg of Evans blue 30 min. before the blood samples were collected. Each point represents the mean of 6 to 10 observations.

calculated from either the dye concentration or the hematocrit was close to 20%.

Plasma volume changes showed a development closely parallel to edema formation. Cutaneous edema started about 40 minutes after injection, reached its peak in 90 minutes and all but disappeared between 3 to 5 hours.

Removal of the adrenals, by itself, does not alter plasma volume significantly. In 9 adrenalectomized rats the mean dye concentration was 115.7 (±2.4) μg/ml with a plasma ratio of 50.4%. The calculated plasma volume was 87.0 ml/kg, not significantly different from the intact group. Under the influence of egg-white, the adrenalectomized rats developed within 30 to 60 minutes edema, cyanosis and irreversible shock. Since most of these animals were dead one hour after the injection of egg-white, study of their plasma volume was done only once, at 45 minutes. The results shown in Fig. 1 indicate a rapid and marked rise of dye concentration and a steep fall of the plasma ratio. The loss of plasma appears to be higher according to the hematocrit readings than according to dye concentration.

Table I shows that plasma loss is related to the dosage of egg-white but, even at the lower dose level the hematocrit gives a higher plasma loss than the dye method. This can probably be explained by the fact that the dye also escapes from the blood together with the proteins to which it becomes bound.

When cortisone was given either to intact or to adrenalectomized rats under the conditions specified above, the plasma volume changes were considerably decreased or even completely prevented. As seen in Table I, 5 mg/kg of cortisone given to intact rats (group 3) prevented the increase in dye concentration, although the hematocrit still indicated a slight plasma loss. Similar results were obtained in adrenalectomized rats pretreated with 10 mg/kg of cortisone (groups 6 and 8). It should be noted that adrenalectomized rats required higher amounts of cortisone to protect them and also that there is a rough proportionality between the dose of egg-white and the concentration of cortisone required for protection.

Microscopic observations. Results shown by direct observations of small blood vessels paralleled the plasma volume changes. These observations extend over a period of 5 hours following the egg-white injection. The intact rats, treated with egg-white, did not show any marked circulatory changes until about 90 minutes after injection. After this interval the flow was slowed down in the dilated and hyperemic metarterioles and arteriovenous capillaries and some clumping of blood cells was seen in the venules. Normal circulation was restored 3 to 5 hours after injection.

In the adrenalectomized animals these changes were both earlier and more severe. Thirty minutes after egg-white injection capillary function was deeply depressed with sludging of blood and stasis. These changes persisted until death of the animals.

TABLE I. Effect of Egg-White on Plasma Volume in Intact, Adrenalectomized and Cortisone-Treated Rats.

No.	From dye conc. Plasma vol.	From dye conc. Plasma loss, %	From hematocrit Plasma vol.	From hematocrit Plasma loss, %	N
1 *Intact rats*, controls	79.5 ± 4.2*	—	79.5 ± 1.7*	—	8
2 Egg-white 2 g/kg	62.3 ± 3.5	22.0	64.0 ± 4.1	19.5	10
3 " " , C† 5 mg/kg	82.8 ± .8	+2.4‡	72.1 ±12.5	9.3	10
4 *Adrenalectomized rats*, controls	87.0 ± 1.8	—	87.0 ± 4.4	—	9
5 Egg-white 1.5 g/kg	68.6 ± 4.8	21.2	54.7 ± 3.3	37.0	7
6 " " , C 10 mg/kg	85.8 ± 4.5	1.0	70.4 ± 9.6	19.2	7
7 " 2 mg/kg	55.8 ± 4.6	35.8	42.6 ± 5.3	51.2	6
8 " " , C 10 mg/kg	73.9 ± 3.2	15.2	58.0 ± 3.0	33.3	6

* Stand. error. † C = Cortisone. ‡ Increase in plasma vol.

Blood samples collected close to peak of reaction: 90 to 120 min. from intact rats, 40 to 50 min. from adrenalectomized animals. Plasma volume values are given in ml/kg body wt. Significance of differences between means was tested with the *t* test. Comparison between groups 2 and 1, 3 and 2, 5 and 4, 7 and 4, 6 and 5, and 8 and 7 gave P values of .01–.001, except for the hematocrit between groups 2 and 1 where the P value was .02–.01.

Cortisone pretreatment was seen to prevent disturbances in capillary circulation. In the intact rats (group 3 of Table I) only slight and transitory red cell clumping was observed. In the adrenalectomized groups treated with cortisone (groups 6 and 8) a slight slowing down of blood flow was also observed.

It should be noted that some clumping of white cells was seen in all groups even in the controls. This was, however, noticeably increased in all egg-white treated animals which have not received cortisone.

Discussion. The purpose of the present studies is to explain the mechanism by which egg-white causes edema and shock. The results obtained also suggest an explanation for the protective action of cortisone. The Evans blue method of plasma volume determination has been examined in critical reviews(8,9) and is considered satisfactory for static evaluations of plasma volume. In the present work the method is used under conditions which introduce at least one more degree of complexity. In acute shifts of fluid, as are produced in the egg-white syndrome, permeability of the vascular wall is increased to protein molecules. Evans blue is known to combine with proteins and will therefore share the fate of these molecules. This may result in a decrease in dye concentration, interpreted as an increased plasma volume.

The same criticism applies to the hematocrit method. If blood cells escape from circulation into the tissues or are stored in reservoir areas the change in plasma ratio may be interpreted as an increased plasma volume. It is possible therefore that the actual plasma loss is greater than indicated by either the dye or the hematocrit methods. With these reservations, it can be stated that the results obtained in this study give a fair indication of gross plasma volume changes. If the changes recorded were smaller their interpretation would be open to doubt.

Plasma loss caused by egg-white was significantly decreased by cortisone. This suggests that cortisone may exert its protective action primarily on the capillary wall and thus maintaining the plasma volume within limits compatible with survival. It is known that when a critical plasma volume is reached a vicious circle is established resulting in irreversible circulatory collapse.

It has been stated(4,5,10) that, although cortisone decreases mortality in adrenalectomized egg-white treated rats, it has no effect on the edema. In all these studies, however, edema was evaluated only by gross inspection by which quantitative changes could not have been detected.

Summary. Egg-white administration to rats results in loss of plasma from circulating blood. In adrenalectomized rats egg-white injection causes a greater loss of plasma than in intact animals. In animals pretreated with cortisone the loss of plasma is significantly smaller than in unprotected animals. The observations suggest that cortisone may act by

preserving capillary function and maintaining plasma volume above the critical level.

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Metachromasia in the Living Cell.* (21015)

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It has been generally agreed that metachromatic granules are not observed in the cytoplasm of fixed fibroblasts(1). They, as well as other cells, however, may show diffuse cytoplasmic metachromasia, which has been shown to be due to RNA(2-4). This might indicate that the metachromatic material of the ground substance(5,6) (not due to RNA), which is presumed to be produced by the fibroblast, acquires the quality of a chromotrope only when outside the cell(1). On the other hand, fixed mast cells show intracellular granular metachromasia. They have been presumed to produce both heparin(7,8) and hyaluronic acid(9-11). Mast cells in tissue culture have been identified by the presence of supravital metachromatic granules(12-17). The purpose of this communication is to describe the presence of metachromatic granules in living cells other than mast cells. It is further shown that their presence does not constitute definitive evidence for the identification of cells as mast cells on this basis alone.

Methods. Toxicity of toluidine blue (ToB), and thionin in low concentrations to culture cells has been found to be small. When ToB was added in a final concentration of 1/200000 to a medium of plasma and 20% embryo extract in chick amniotic fluid at the time of explantation, growth observed for 4 days was not inferior to growth in control cultures. Easily observable intracellular metachromasia, however, was not produced until dye concentrations of 1/50000 to 1/100000 were reached, when some toxicity to cells resulted. These concentrations, however, offer the best opportunity for observation of metachromasia and were employed in these studies. Fibroblasts from human skin and from chick skin and heart were cultured in a liquid medium of 20% embryo extract in chick amniotic fluid (18), using the coverslip method. At the second to fourth day of cultivation, the medium was changed to one of 1/50000 ToB in Ringer's solution. A dye concentration of 1/40000 ToB was used when cultures were grown in a plasma medium. Under these conditions, on direct microscopic observation with high power magnification, vital staining started at one side of the fibroblast near the nucleus. First a faintly rose-stained spot appeared in which tiny pink granules were embedded. The granules soon became more distinct and gradually, close to them, violet- or purple-colored granules appeared; the latter

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subsequently came to occupy larger areas on both sides of the nucleus, sometimes filling the whole cytoplasm. The number of rose-stained granules then decreased and often disappeared altogether. The purple of the granules gradually became more intense but many of them could still be seen embedded in a rose-stained matrix. The healthier the appearance of the cells, the longer the rose-colored granules persisted. When nontoxic low concentrations of ToB were used, *i.e.*, below 1/100000, the dye was excreted and the cells continued to grow. When higher concentrations were used, after hours or days, a light blue color first of the nucleoli, then of the nucleus and cytoplasm indicated the approaching cell death. Diffuse orthochromatic staining is evidence of cell death, whereas metachromatic granular staining of the fibroblast is one more criterion of cell viability. Epithelial cells from stomach, intestine, and kidney also showed supravital granular metachromasia, which thus appears to be a general phenomenon in living cells and not connected solely with mesenchymal cells.

Results. Metachromasia in the living cell was influenced neither by pretreatment of the fibroblasts with hyaluronidase† (75 TRU/ml), nor by adding hyaluronidase (75 TRU/ml) to the culture medium. There is no certainty, however, that the enzyme reached the cell interior. Cytoplasmic vacuoles experimentally produced by slight hypotonicity of the medium stained metachromatically with ToB.

When the washed culture of fibroblasts was placed in 0.04 molar NaCl solution, no granular staining with neutral red was seen (19). Similarly in such a medium containing ToB, no metachromasia occurred but, like vital staining with neutral red, it could be produced by increasing the salt concentration of the medium after 10 to 30 minutes of exposure to the hypotonic medium. This would indicate that granular vital staining as well as vital granular metachromasia depends upon an active process in the cell, which could concentrate the dye only from a solution containing a certain concentration of neutral salts. It seems worth mentioning that both metachromatic vital granules and secretory granules first appear close to the nuclear membrane and subsequently diffuse into peripheral parts of the cytoplasm.

When high concentrations of ToB, incompatible with cell life (about 1/5000), were added to living cells in tissue culture, diffuse rose staining of dead cells ensued. Similarly, when orthochromatically stained nonliving cells in tissue culture were subsequently treated with a high concentration of ToB, the blue color of the cells promptly changed to bright rose. Thus, in the dead cell, metachromasia was produced by increasing the dye concentration. In the living cell, variations in dye concentration, in the limits compatible with cell life, change only the speed of the appearance of vital metachromasia. While in nonliving cells changes in pH of highly concentrated ToB solutions lead to loss of metachromasia on the acid side, and increased metachromasia on the alkaline side, pH ranges in the culture medium compatible with cell life have, as a rule, no direct influence on vital metachromasia.

Discussion. It is possible that the action of metachromatic dyes on nonliving cells in tissue culture is, to a certain extent, comparable with their action in model experiments *in vitro,* whereas, with vital metachromasia, factors present only in the living cell are involved. Therefore, once produced, vital granules (*e.g.*, the "crinome") do not subsequently stain metachromatically in dead cells because granular vital metachromasia is dependent upon a continuous process in the living cell.

If we assume that in the living cell dye accumulation may be achieved by an active process in the cell(19-21,26) the present data would be consistent with the view that intracellular segregation of dye and its concentration(22-25) may be a factor sufficient to produce metachromasia. The regular shift, however, from pink towards saturated purple (a saturated purple is produced by a mixture of colors from both ends of the visible spectrum) in the course of vital staining with ToB and the shift from purple to blue at cell death would seem to involve, besides increased density of the granules(23,26-28), possibly

† Hyaluronidase—Wyeth.

also more complicated processes, which at the present time are unexplained. Further work on the mechanism of this process is contemplated.

It appears that, in tissue culture, mast cells (12-15) cannot be specifically identified by supravital metachromasia since the living cells we have examined in tissue culture have all stained metachromatically.

Summary. 1. Intracellular granular metachromasia, appearing in a sequence of two colors, *i.e.*, pink and purple, is a general phenomenon found in a variety of living cells in tissue culture. 2. In tissue culture cells, the appearance of intracellular granular metachromasia is evidence of cell viability and intracellular orthochromasia, one more criterion of cell death. 3. Mast cells, or "precursors" of mast cells cannot be specifically identified by supravital metachromasia. 4. Dye condensation effected by an active process in the cell, as one possible factor in producing vital metachromasia, is discussed.

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Experimental Production of Combination Forms of Virus.* IV. Mixed Influenza A-Newcastle Disease Virus Infections. (21016)

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Previous papers from this laboratory(1,2) have established the fact that when two strains of influenza virus (A-A or A-B) are inoculated together into the allantoic sac, some of the progeny therefrom may have antigenic characteristics of both parent types. This doubly antigenic virus was called X virus and was recognizable by the fact that it reacted with specific antisera of both parent types in the hemagglutination inhibition (HAI) and neutralization tests. The fact that two antigenically unrelated viruses could participate in forming the coat of single particles led us to study combined Influenza A-Newcastle Disease Virus infections. The following account shows that these two strains can contribute to the antigenic make-up of single particles.

Materials and methods. The Melbourne strain of influenza A and the Beaudette strain of Newcastle Disease Virus were used throughout this study and are referred to as M and N. All antisera were prepared in rabbits and before use in HAI tests were treated with RDE and absorbed with Lee virus(3) until non-specific inhibitors were completely removed. Untreated sera were used for *in ovo* neutralization tests. In all HA (hemagglutination) and HAI tests, the virus used was first treated with sodium periodate which had a stabilizing effect on the NDV hemagglutinin and this was essential in order to get valid and reproducible results with this virus. To 0.5 ml of infected allantoic fluid was added 0.1 ml of M/10 $NaIO_4$. After 30 minutes at room temperature, 0.1 ml of 40% glucose was added. This treatment was applied routinely to all fluids, many of which contained mixtures of M and N. It did not lower the HA titer of either agent. HA and HAI tests were carried out by the pattern method(1). Virus titrations *in ovo* were usually done with 3-fold, sometimes 10-fold, dilution steps, and 10 eggs per dilution. Incubation for 40 hours was followed by testing the allantoic fluids for HA after treating them with periodate.

Search for mixed antigenic particles with the HAI test. The M and N strains were found to have very similar growth rates in the allantoic cavity (roughly 30 hours for a maximum HA yield from 1 ID_{50}) and, hence, there was no consistent tendency for one particle to outgrow the other in mixed infections. Two sets of experiments were carried out initially, both with mixed inocula. In the first, the number of particles injected gave a particle/host cell ratio of more than one, while in the second experiment the input was small, about one ID_{50} per egg. 1) For the large inoculum, both viruses were concentrated 100 times from allantoic fluid by centrifugation. Each concentrate was diluted in 10-fold steps and various M-N pairs of these dilutions were made by combining equal volumes and these mixtures were inoculated into 5 chick embryos each. After 24 hours' incubation at 37°C, the fluids were harvested and tested for the type of the predominating virus by means of the HAI test. 2) Mixed infections were also carried out with dilute inocula. Each virus was diluted by twofold steps to the point where each 0.1 ml contained about one ID_{50} and, again, various combinations of dilutions were made and inoculated into eggs. After 40 hours' incubation, the fluids were harvested and tested for the predominant serotype by the HAI test. The results are shown in Table I.

In general, the virus type which predominated in the inoculum also predominated in the final yield, whether the total amount injected was large or small. However, among the eggs that received nearly equal amounts of the two infecting strains, were a number that yielded fluids containing HA that was not inhibited by either parent antiserum. This was very different from what has been obtained with

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TABLE I. Simultaneous Inoculation of Allantoic Sac with an Influenza A and a Newcastle Disease Virus.

Exp. No.	Inoculum total ID_{50}, log		Ratio ID_{50} inoculated, Mel/NDV	Predominant virus type in fluid after incubation				
	Mel	NDV						
1	5.95	8.53	1/400	N	N	N	N	
	6.95	8.53	1/40	N	N	N	++	++
	7.95	8.53	1/4	N	++	++	++	++
	8.95	8.53	3/1	M	M	M	M	M
	8.95	7.53	30/1	M	M	M	M	
2	.3	1.3	1/10	M	N	N	N	N
	.5	1.3	1/4	M	N	N	++	
	1.0	1.3	1/2	M	++	++		
	1.3	1.3	1/1	M	M	M	M	++
	1.3	1.0	2/1	M	M	M	M	++
	1.3	.5	4/1	M	M	M	N	
	1.3	.3	10/1	M	M	M	M	

Both viruses were inoculated together in 0.1 ml. After 24 (Exp. 1) and 40 (Exp. 2) hours of incubation, allantoic fluids were removed and tested for the predominating type of virus by means of the HAI test, in which 4 HA units were tested against a 1/64 dilution of NDV and Mel antiserum. M indicates predominance of the Melbourne strain, N of Newcastle Disease Virus, and ++ indicates that the two agents were present in equal (HA) or nearly equal titer.

A-A and A-B influenza virus infections, which yielded fluids containing an HA that was inhibited by both parent type antisera. This behavior (no inhibition) is what one could expect from a simple mixture of equal amounts of the 2 parent viruses and this similarity was confirmed by further tests. HA titrations on these fluids done in anti-M and anti-N sera showed that the M and N HA titers were equal and that added together they equalled the total HA titer. There was no titer discrepancy in these fluids and, hence, no evidence of the presence of doubly antigenic or X virus.

Some of these fluids in which the HA titers were equal for M and N (++ fluids) were passed to other eggs in an attempt to pick out an X form. When fluids from individual eggs in titrations were tested for the predominant serotype, ++ fluids were found in eggs that received low as well as high dilutions. It was not necessary to use a large inoculum to perpetuate mixed infections and ++ fluids were found in a number of passages, even when the inoculum contained just a few ID_{50} of virus. A few characteristic passages are shown in Table II. It appeared that M and N could be readily propagated together indefinitely.

The results up to this point failed to give any indication of the occurrence of virus particles having mixed NDV-influenza A antigens, but the evidence showed that in carrying the 2 together equivalent yields of the 2 viruses from single eggs were not uncommon. Previously discovered X forms showed their mixed character by *in ovo* as well as by *in vitro* tests and for this reason a number of ++ fluids were examined for the ability of the virus to be neutralized by both parent antisera.

Demonstration of X virus with in ovo neutralization. A number of ++ fluids from various passages of the M-N combination were tested for neutralization by M and N sera. Each fluid was diluted in threefold steps, and normal and M and N immune sera (1/32) were each added to one set of dilutions. The results are shown in Table III. Fluids 7 and 8 were controls which consisted of artificial mixtures of the M and N strains. The number of ID_{50} of M and of N virus failed to add up exactly to the total number of ID_{50} in each fluid, but the differences were small and could be attributed to the inherent errors of *in ovo* titration.

Some of the ++ fluids showed no significant titer discrepancy(1,2) by these methods but at least half of those tested showed double neutralization of such a degree that there was no doubt that quite a high proportion of particles with a mixed antigenic character were present. Fluid No. 6, for example, showed a

TABLE II. Serial Passage of Influenza A and NDV Together in the Allantoic Sac.

Dilution of AF inocu- lated, log	Passage No. 1				4					7					11				
0	++	++	++	++	M	++	++	++	++	++	++	++	++	++					
−1					N	++	++	++	++	++	++	++	++	++					
−2					++	++	++	++	++										
−6	M	++								M	M	++	++	++	N	++	++	++	++
−7	M	N	++		M	++	++	++	++	M	++	++	++	++	N	N	N	N	N
−8	M	N	N		M	M	M	N	N	M	N	N	N	++	N	N			
−9					M	N				M	N				M				

Fluid from individual eggs was tested for predominant virus type as in Table I. M indicates predominance of Melbourne strain, N of Newcastle Disease Virus, and ++ indicates that the two agents were present in equal or nearly equal titer. Transfer from one passage to another was done with ++ material from a 10^{-6} dilution or higher.

reduction in titer of log 1.2 with both antisera. This could hardly have been due to chance since a difference of log 0.6 is ordinarily considered significant, even when the dilutions are made in 10-fold steps. Doubly neutralizable fluids were obtained from a number of different passages. Since these sera were quite specific, it is felt that these results provide excellent grounds for the assumption that doubly antigenic influenza-NDV particles were present.

Inability of M-N Type X particles to initiate mixed infections. The occurrence of virus particles with surface antigens from 2 parents raised the question of whether or not such particles might not individually give rise to both parent serotypes. This question had already been investigated with A-B X forms by a technic which indicated that those particles were of a single parental genotype(2). The test was applied to M-N type X particles with the same result. The primary step, as with other virus combinations, was to determine the frequency of mixed infections in individual eggs after the inoculation of limiting infectious dilutions of *in vitro* mixtures of M and N viruses. These 2 strains were mixed in equal amounts (in terms of ID_{50}) and 2-fold dilutions of the mixture were made between 10^{-8} and 10^{-10}. Forty eggs were inoculated with each dilution. After incubation, all of the fluids which contained detectable amounts of HA were tested for the predominant serotype by the HAI test. An attempt to isolate a second type was made by inoculating the fluids into one or 2 eggs in the presence of antiserum against the predominant strain. The number of mixed infections found is shown in Table IV and in Fig. 1.

In Fig. 1, the dotted line shows the average frequency of mixed infection from 4 previous similar tests with A-A and A-B combinations (2). It will be seen that the frequency of mixed infection with artificial mixtures of M and N was a little less but of the same order of magnitude. It is fairly certain, therefore, that mixed infection was a chance event, dependent on the accidental inclusion of 2 particles of opposite serotype in one inoculum.

A single X fluid of the M-N type was selected for further study. This fluid contained

TABLE III. *In Ovo* Infectivity Titrations on ++ Fluids Done in Presence of Normal and Immune Antisera.

Fluid No.	Passage No.	Dilution of fluid used for infecting egg	ID_{50} of virus tested in presence of serum: Normal	Anti-M	Anti-N	% of virus neutralized by both sera (X virus)
1	1	−8	9.35	9.18	8.63	13
2	6	−6	8.38	8.20	7.66	10
3	4	0	9.07	8.64	7.55	60
4	1	−7	9.75	9.06	8.79	69
5	10	−5	9.44	8.75	8.65	74
6	8	−8	9.64	8.42	8.38	89
7	Control mixture		9.85	9.50	9.11	37
8	" "		9.91	9.54	9.50	19

Fluids were all taken from experiments like those shown in Table II. All were ++ in type. Infectivity titrations were done *in ovo* using 3-fold dilutions and 10 eggs per dilution. Second and third columns of titrations above are the N and M titers respectively. These were added together and subtracted from the first column, the remainder being titer deficiency indicated the amount of virus neutralized by both sera (X virus).

74% X virus as determined by *in ovo* titration and it was inoculated into eggs at high dilution. The number of mixed infections which were found in individual eggs is shown in Table IV and Fig. 1. The ability of these X particles to give rise to mixed infection was as low as the control mixtures and, hence, there is no reason to assume that they possessed intrinsic capability of inducing mixed infection.

Discussion. The foregoing results may be summarized by saying that they are essentially the same as those found with mixed A-B infections, the only exception being that the mixed antigenic character of the M-N X form could be demonstrated by neutralization only and not by hemagglutination inhibition. This is clearly a minor difference. X virus has now been demonstrated with numerous A-A, several A-B, and a single A-NDV combination. The difficulties of demonstrating X virus by the *in vitro* test, increased in that order, and the HAI test failed to reveal these forms with A-NDV combinations. Yet X virus was

TABLE IV. Yield of Single and Mixed Infections in Individual Eggs Inoculated with Small Amounts of Virus.

Exp. No.	Inoculum	Dilution of inoculum	No. of eggs inoculated	No. of eggs infected	No. eggs infected with M and N	% of eggs infected	% infected eggs mixedly infected
1	M and N viruses, artificially mixed in ratio 1/3	8.0	39	39	28	100	72
		8.3	38	31	6	82	19
		8.6	40	18	3	45	17
		8.9	39	17	0	44	0
		9.2	40	7	0	17	0
		9.5	40	2	0	5	0
		9.8	39	0	0	0	0
2	M and N viruses, artificially mixed in ratio 1/2	8.6	40	34	15	85	44
		8.9	40	16	5	40	31
		9.2	40	12	0	30	0
		9.5	40	10	0	25	0
3	X fluid M-N type from passage 10. 74% X virus by neutralization	8.0	39	26	2	67	8
		8.3	40	17	2	42	12
		8.6	40	6	0	15	0
		8.9	40	3	0	7	0
		9.2	40	0	0	0	0

Each infected egg was tested for the predominant serotype by the HAI test and the fluid was then inoculated into 2 eggs in the presence of a serum to suppress the dominant organism, in order to find a second type if present. Infectivity titers for each virus were calculated separately in Exp. 1 and 2 and these were used to determine the ratios M/N.

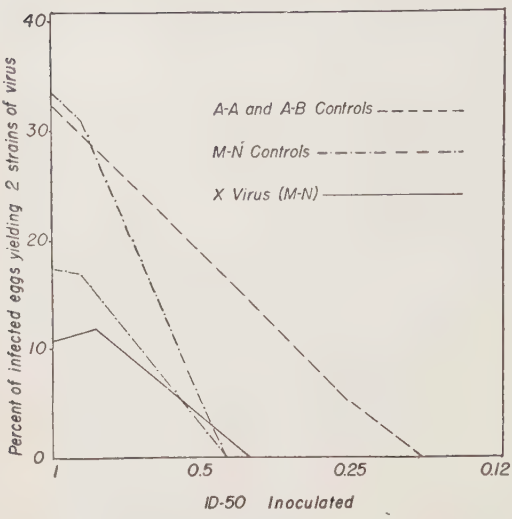

FIG. 1. Effect of number of ID_{50} inoculated on % of infected eggs that yielded 2 strains.

readily demonstrated in all 3 types of mixtures by the *in ovo* neutralization test.

In several previous communications from this laboratory, it was maintained that the occurrence of X_1 virus could best be explained by the phenomenon of phenotypic mixing(4, 5,2). Burnet, *et al.* alluded to X virus (A-A type) as a kind of recombinant(6) but later on discarded this idea in favor of heterozygosis, diploidy, or some type of doublet formation as alternate explanations(7). It is a confusing fact that with A-A infections, X particles and particles heterozygous for antigenic type are found in the same eggs(2). This would lead one to suspect that the two phenomena were connected were it not for the absence of heterozygosis from A-B and A-NDV infections.

The belief that a mixed antigenic condition of the virus surface is due to phenotypic mixing was suggested and supported by phage experimentation(8,9), in which it was shown that the combination of 2 kinds of genotypes with several tail antigens was an almost random process. Luria, in summarizing this work from his laboratory, suggested that one of the implications of phenotypic mixing was a 2-stage process of phage maturation, the first a synthesis of specific materials followed by a less specific assembly of these substances into finished particles(10). He suggested that the phage DNA in the infected cell may induce the formation of specific antigens while in an extended state. The assembly of phage antigens around the condensed phage DNA cores would then follow as a separate step.

Summary. Mixed infection with the viruses of influenza A and Newcastle disease virus was found to result in the formation of some particles which had antigenically mixed surfaces, *i.e.*, which contained components from both parent types. The phenotypic change was transitory and did not appear in the progeny of such a particle. It is suggested that this phenomenon is due to phenotypic mixing.

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Coproporphyrin Studies in Children. 2. Erythrocyte Coproporphyrin and Protoporphyrin Levels in Normal Infants and Children.* (21017)

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Although the presence of free protoporphyrin in the circulating red blood cells was described by van den Bergh and Hyman(1) in 1928, it is only with the advent of improved fluorimetry that coproporphyrin has also been found in the red blood cells by Schwartz and Wikoff(2). In studies on normal adults, Watson(3) has reported the mean erythrocyte protoporphyrin (EP) to be 28.5 ± 4.5 μg/100 cc of packed erythrocytes for males and 41.7 ± 5.9 μg/100 cc for females. The mean erythrocyte coproporphyrin (ECP) was found to be 0.3 ± 0.39 μg/100 cc of packed erythrocytes for males and 0.7 ± 0.6 μg/100 cc for females. Krammer and his coworkers(4) have shown essentially the same values for EP, but their ECP values were 0.76 ± 0.26 μg/100 cc of packed erythrocytes for males and 0.71 ± 0.23 μg/100 cc for females. They attribute this difference of ECP concentration to the greater sensitivity of the fluorimeter used.

Since EP and ECP are known to be altered in many childhood diseases(3-8), the present study was undertaken to define the range of EP and ECP in normal infants and children.

Methods and materials. A total of 24 newborn infants and 19 older children of various ages were selected for this study. All were healthy and free from anemia and other hematologic disturbances. The following studies were done on samples of venous blood collected in potassium oxalate tubes: 1) hemoglobin, 2) reticulocyte count, 3) total plasma bilirubin(9), 4) EP and ECP of the packed erythrocytes using the method of Schwartz and Wikoff(2) with certain minor modifications. The coproporphyrin was measured on a Calectron fluorimeter† employing a photomultiplier tube and using the Corning No. 5543 as primary filter and Corning No. 2418 as the secondary filter. Values were determined by comparison with standard coproporphyrin solutions in 0.3 N HCl.‡ The protoporphyrin concentration determined by absorption analysis in an Evelyn colorimeter using a calibration curve as shown by Grinstein and Watson(10).

Results. The EP and ECP for normal children are given in Table I. The mean EP was 66 ± 37 μg/100 cc packed erythrocytes and the mean ECP was 1.1 ± 0.57 μg/100 cc packed erythrocytes. The EP and ECP did not appear to be related to either the age or sex of the children.

The EP and ECP for normal newborn infants are given in Table II. The mean EP was 86 ± 37 μg/100 cc packed erythrocytes and the mean ECP was 1.5 ± 0.70 μg/100 cc packed erythrocytes. The EP and ECP did not appear to be related to the sex, weight, or

TABLE I. Erythrocyte Coproporphyrin and Protoporphyrins in Normal Children.

Sex	Age	Hgb (g %)	Retic. (%)	EP (μg/100 cc)	ECP (μg/100 cc)
♀	3 wk	12.0	.3	54	1.3
♀	3 yr	13.2	.4	43	.6
♂	3	12.0	.2	150	1.8
♀	5	16.6	.3	175	2.7
♂	5	14.2	.3	92	.7
♀	5½	12.1	.5	47	.7
♂	6	11.6	.6	78	.5
♂	6	13.8	.5	59	.8
♀	6	13.4	.8	55	1.7
♂	7	13.4	.5	55	.8
♂	8	13.1	.6	69	1.8
♂	10	12.9	.4	33	.7
♂	10	13.1	.3	30	.7
♀	12	12.9	.3	58	1.8
♂	12	12.5	.5	38	1.1
♀	12	14.2	.3	22	.7
♀	13	13.1	.3	68	.8
♀	15	13.4	.4	58	1.6
♀	15	15.7	.2	62	1.3
			Mean	66	1.1
			S.D.	±37	±.57

Plasma bilirubin was less than .8 mg % in all instances.

* Aided by grants from the M&R Dietetic Laboratories and the Playtex Park Research Institute.

† Manufactured by Calectron, Inc., St. Paul, Minn.

‡ Kindly supplied by Dr. Samuel Schwartz.

TABLE II. Erythrocyte Coproporphyrin and Protoporphyrin in Newborn Infants.

Sex	Wt (lb)	Hgb (g %)	Bili. (mg %)	Retic. (%)	EP (μg/100 cc)	ECP
			Cord blood			
♂	7'11	16.6	1.3	2.7	125	1.8
♂	7'3	16.0	4.3	2.8	203	2.8
♂	6'14	16.6	1.9	1.8	167	.8
♂	7'2	17.1	.8	2.1	101	.6
♂	8'1	18.1	.6	1.6	63	1.4
♀	7'	20.1	3.7	1.8	114	1.4
				Mean	125	1.5
			First day (0-24 hr)			
♀	7'14	18.9	.9	3.1	63	1.3
♂	8'8	23.8	1.6	2.3	102	1.9
♂	5'12	18.1	3.2	2.6	46	1.3
♂	6'11	17.1	3.6	2.4	96	3.1
♀	7'11	22.0	1.6	2.1	69	1.6
♂	6'11	22.0	4.3	2.9	54	2.4
				Mean	71	1.9
			Second and third day (24-72 hr)			
♂	7'13	17.0	1.4	1.0	55	.9
♀	7'7	17.1	3.2	1.8	50	1.6
♀	6'14	19.9	0	2.6	107	1.6
♀	6'14	19.6	5.0	1.4	95	2.1
♂	9'2	14.9	5.8	3.6	97	2.6
♀	8'5	20.2	1.9	2.1	70	.9
				Mean	79	1.6
			Fourth and fifth day (72-120 hr)			
♂	8'7	17.0	3.6	.8	96	1.7
♀	6'6	21.0	1.0	1.4	68	1.1
♀	7'10	19.6	1.6	1.3	54	1.7
♂	8'6	19.3	2.6	1.0	48	.2
♂	6'10	13.4	.9	.8	54	.8
♂	8'4	19.9	.5	.5	67	.7
				Mean	64	1.0
			Mean for infants		86	1.6
			S.D.		±37	±.7

hemoglobin determination. The correlation coefficient between bilirubin and ECP was $r = +0.62$ and between bilirubin and EP was $r = +0.22$. Although no significant difference could be found between the EP and ECP values from the cord blood to the fourth and fifth day, there appeared to be a decreasing trend with the values on the fourth, and fifth day close to normal.

The correlation coefficient in the entire series between ECP and reticulocyte percentage was $r = +0.43$ and between EP and reticulocyte percentage was $r = +0.27$. The correlation between EP and ECP was $r = +0.40$.

Discussion. The results show a general increase of both EP and ECP among the newborn infants and older children as compared with normal adults(5,6). The increase in EP in the older children is highly significant ($P < 0.01$) when compared with normal adults, but the difference between the newborn infants and older children is not significant. The increase in ECP in children is not significant when compared with normal adults, but the ECP in newborn infants shows a value greater than 2 standard deviations above the mean for the older children and is highly significant ($P < 0.01$) when compared with normal adults.

The increase of EP in infants and children probably reflects the iron deficient state in general. Cartwright(11) showed in adults that iron deficiency is manifested by decrease in serum iron, a reciprocal rise in serum copper, and moderate elevation of the erythrocyte protoporphyrin. Sturgeon(12) has suggested in infants and young children that the hypoferremia with reduction in the per cent saturation of the total iron-binding capacity, hypercupremia, and elevated erythrocyte protoporphyrin represents a general state of iron deficiency. The data here confirm his observations.

The increase of ECP in the early newborn period probably reflects increased erythropoiesis *in utero*. Schwartz and Wikoff(2) have shown that a close correlation exists between ECP and reticulocyte percentage. By removing large volumes of blood at regular intervals from dogs, they were able to stimulate erythropoiesis and cause simultaneous increase of reticulocytes and ECP levels. They believe that ECP is therefore a reflection of increased erythopoiesis in the body. Goldbloom and Gottlieb(13,14) first suggested that the fetus acquires an overabundance of red cells as a response to the relatively anoxic conditions *in utero*. At birth, these extra cells are no longer needed and are destroyed. Mollison(15) has shown that the rate of breakdown of the newborn infant's own erythrocytes may be increased during the first 10 days of life. Hsia(16) has found that the mechanical fragility of erythrocytes is elevated at birth and falls to normal levels by the fifth and sixth days of life. He found a direct correlation between the height of the mechanical fragility of erythrocytes on the first day

and the subsequent degree of hyperbilirubinemia suggesting an increased breakdown of "abnormal" erythrocytes during that period.

In the present study, the correlation between reticulocyte percentage and ECP described by Schwartz and Wikoff(2) could only be partially demonstrated. This is not surprising since very few of the reticulocyte counts exceeded 2% and it is difficult to demonstrate real differences at this low level. However, in the infants with high ECP, where presumably there is an increased degree of erythropoiesis prior to birth, there is a greater destruction of erythrocytes and subsequently more jaundice. In those with low ECP, the converse could be true. Sturgeon(12) has pointed out the extreme range and variability of EP in children as compared with normal adults. This is confirmed for both EP and ECP in this study. From the practical standpoint, it would appear as has been previously suggested in connection with urinary coproporphyrin excretion(17), that values higher than twice the standard deviation above the mean for either EP or ECP or both should be regarded as abnormal.

Summary. 1. Erythrocyte coproporphyrin and protoporphyrin levels have been determined in normal newborn infants and older children. 2. There is a significant elevation of EP in children as compared with normal adults. This increase probably reflects the general iron deficient state in children. 3. There is a significant elevation of ECP in newborn infants as compared with normal adults. This increase probably reflects increased erythropoiesis *in utero* and rapidly disappears by the fourth and fifth day of life. 4. The EP and ECP should be regarded as abnormal if they exceed twice the standard deviation above the mean.

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Effect of Ovariectomy, Adrenalectomy and Hypophysectomy on Growth of Spontaneous Mammary Tumors in Mice.* (21018)

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It is well known that alterations of hormonal stimulations may change the incidence of mammary tumors in susceptible mice. However, the effects of the surgical removal of some endocrine glands (ovaries, adrenals, hypophysis) upon the growth of established spontaneous mammary tumors, as well as on the survival time of mice bearing them and submitted to the above mentioned operations, have not been conclusively established. Baatz(1) reported that ovariectomy does not alter the growth of established spontaneous mammary tumors in mice. Gardner(2) found in mice bearing spontaneous breast tumors, that hypophysectomy performed during pregnancy or immediately after delivery did not change the growth of the tumors and also did not cause regression of the hyperplastic nodules which are considered as precancerous lesions. In humans, however, with carcinoma of the breast, favorable results have been reported following the surgical removal or X-radiation of the ovaries, bilateral adrenalectomy or both combined operations (see review by Nathanson and Kelley(3)). Recently Luft (4) has reported the results obtained in 30 patients with carcinoma of the breast treated by the removal of the pituitary gland. In 10 of those patients hypophysectomy did not change the course of the disease and they died early after the operation. In 10 cases the tumor development was only retarded and in 10 cases there was a dramatic subjective and objective improvement in the disease, namely disappearance of pain, regression of metastic lesions and remarkable effects on the general condition of the patients.

The present experiments deal with the effect of bilateral ovariectomy alone, or combined with bilateral adrenalectomy, and the effect of hypophysectomy alone and combined with ovariectomy and adrenalectomy upon the growth of spontaneous mammary tumors in mice. Also the survival time after the various types of operations was recorded.

Methods. Mice of different strains and hybrids bearing spontaneous mammary carcinomas of a size ranging between 5 and 14 mm were used. Bilateral ovariectomy as well as adrenalectomy was performed through a lumbar incision of the skin and muscular wall. Hypophysectomy was done following the technic described by Thomas(5). In all the groups in which adrenalectomy was done, 1% NaCl solution was given in the drinking water and cortisone acetate was injected subcutaneously once every other day in a dose of one to 5 γ per mouse. Once or twice a week adrenal cortical extract in a dose of 0.1† cc per mouse was injected subcutaneously. Body weight and tumor size were recorded 2 or 3 times a week. Tumor size was measured with calipers, taking the 2 largest diameters of the tumor and the size was arbitrarily calculated according to the formula $S = \sqrt{axb}$, a and b being the 2 major diameters.

Results. The results are summarized in Table I. It can be seen that there was no tumor regression either in the untreated or in the ovariectomized group. In fact all tumors in both groups continued to grow and killed the mice in 53 and 52 days after the operation, respectively.

Bilateral adrenalectomy alone produced in one out of 30 mice a complete regression of the tumor; 3 showed a temporary regression; 6 showed neither increase nor decrease of the tumor size and in 23 the tumor grew pro-

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† Equivalent to 5 dog units (Eschatin, Parke, Davis & Co.)

TABLE I. Effect of Ovariectomy, Adrenalectomy and Hypophysectomy upon the Growth of Spontaneous Mammary Tumors.

Operation*	No. of mice	Complete regression‡	%	Partial regression	%	No change	%	Increase	%
0	22	0		0		0		22	100
†								53	
Ov.	20	0		0		0		20	100
†								52	
Adr.	30	1	3.3	3	10	6	20	20	66.6
†		23		10		13		18	
Adr. & Ov.	27	1	3.7	2	7.4	0		24	88.8
†		28		16				17	
Hyp.	26	3	11.5	5	19.2	3	11.5	15	57.7
†		34		14		12		22	
Hyp., Ov. & Adr.	5	0		1	20	1	20	3	60
†				10		10		8	

* Ov. = Ovariectomy; Adr. = Adrenalectomy; Hyp. = Hypophysectomy.
† Avg survival time in days.
‡ Complete regression of tumor was confirmed histologically.

gressively. The survival time after the operation was as follows: 23 days for the mouse showing complete regression of the tumor; 10 days for the group showing a temporary regression; 13 days for those in which the tumors did not change in size and 18 days for those in which the tumors grew progressively.

Simultaneous removal of both adrenals and ovaries produced 1 complete regression out of 27 mice; 2 temporary regressions and 24 in which the tumors grew progressively. The survival time was 28 days for the mouse in which the tumor regressed completely; 16 days for those showing a temporary regression and 17 days for those in which the tumors grew after the operation.

In the group of hypophysectomized mice, 3 out of 26 animals showed complete regression of the tumor; 5 showed temporary regression; in 3 the tumor did not change in size and in 15 the tumors grew progressively.

The survival time after the operation was 34 days for those in which the tumor regressed; 14 days for those showing temporary regression; 12 days for those in which there was no change in the tumor size and 22 days for those showing progressive growth of the tumor.

Finally, in the small group of mice in which the 3 operations (ovariectomy, adrenalectomy and hypophysectomy) were performed, only 1 mouse out of 5 showed a temporary regression of the tumor; in 1 the tumor did not change in size and in 3 there was an increase in tumor size. The survival time in this group ranged between 8 and 10 days.

Fig. 1 shows the mean body weight changes in different groups of mice after the operation. As can be seen, the hypophysectomized as well as the adrenalectomized mice showed a considerable decrease in body weight. However, in the group of adrenalectomized and gonadectomized animals there was practically no change in the mean body weight after the operation.

Discussion. The above mentioned results indicate that as far as the growth of already established spontaneous mammary tumors is concerned, the removal of both ovaries did not produce any appreciable change in the rate of growth. In fact, all tumors grew after the operation and killed the host in a time comparable to the unoperated controls.

Bilateral adrenalectomy alone and combined with ovariectomy produced complete regression of one tumor in each group (3.3% and 3.7% respectively), few tumors showed either a temporary regression or no change in size, but most of the tumors in both groups (66.6% and 88.8% respectively) grew progressively in spite of the absence of the adrenals or of both adrenals and ovaries.

The group in which hypophysectomy alone

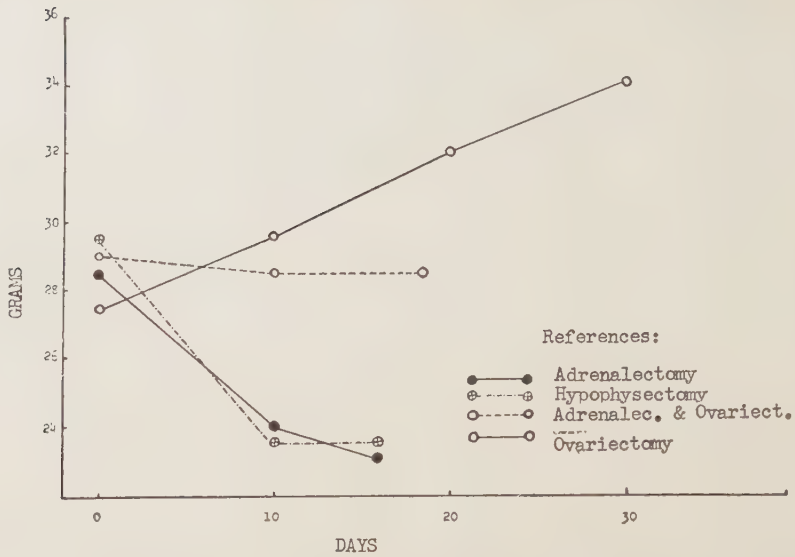

FIG. 1. Mean body weight changes in mice bearing spontaneous mammary tumors submitted to ovariectomy, adrenalectomy, ovariectomy-adrenalectomy and hypophysectomy. Operation at time 0.

was performed showed a higher incidence of complete tumor regressions (11.5%), few tumors showed either a temporary regression or no change, but again most of them (57.7%) continued to grow after the operation, in spite of the absence of the pituitary gland.

The number of mice in the group in which the three operations were performed (ovariectomy, adrenalectomy and hypophysectomy) was too small to permit definite conclusions. In fact, the removal of the 3 glands produced a very high mortality incidence at 12 to 24 hours after the operation.

Experiments are already being undertaken to find out whether tumors showing a temporary regression or no change, would show any alteration in their behavior when transplanted into a new intact, adrenalectomized or hypophysectomized hosts.

Since most of the adrenalectomized or hypophysectomized mice showed a significant decrease in body weight after the operation, it is rather difficult to decide whether the tumor changes obtained are due to the loss of body weight *per se* or to the absence of the glands. It is well known that a reduction in food intake may change the incidence and growth of spontaneous and transplanted mammary tumors, but the fact that most of the tumors grew in spite of the loss in the body weight might be an indication that perhaps inanition was not the only factor responsible for the changes in tumor size obtained.

As far as the survival time after the operation is concerned it is quite clear that in the groups in which either adrenalectomy or hypophysectomy was performed, it was significantly shorter than in the control or ovariectomized groups. The old age of the animals, the presence of the tumors and the difficulty in maintaining the mice upon a properly adjusted replacement therapy might account for the shorter survival time found even in those mice in which there was a complete regression of the tumor.

Summary. 1. The effect of ovariectomy, adrenalectomy, and hypophysectomy upon growth of spontaneous tumors in mice has been studied. 2. Bilateral adrenalectomy alone or combined with ovariectomy produced a complete regression of the tumor in 3.3% and 3.7% of the mice respectively. Hypophysectomy was followed by regression in 11.5% of mice. 3. All groups in which either adrenalectomy or hypophysectomy was performed, showed a shorter survival time than unoperated controls, even in those mice showing a complete regression of their tumors.

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Level of C-Reactive Protein as a Measure of Acute Myocardial Infarction.* (21019)

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The C-reactive protein (CRP) is an abnormal constituent of human serum(1). It is in all likelihood an alpha globulin which is formed by the body in response to an inflammatory reaction(2-4). It is called C-reactive protein because it forms a precipitate with the somatic C-polysaccharide of the pneumococcus(5). Small amounts of this protein may be detected in human serum by a precipitin test using a specific antiserum from rabbits hyperimmunized with purified C-reactive protein(6,7).

Previous studies have shown that a positive test for CRP is a non-specific but sensitive indicator of inflammation of infectious or non-infectious origin(3,4). Anderson and McCarty(8), Stollerman and coworkers(9), and the authors(10) have employed the CRP test for the detection of low-grade inflammation in rheumatic fever. Since myocardial infarction in coronary artery disease is also associated with an inflammatory response, it was felt that the CRP test might be useful in determining the presence of inflammation associated with myocardial infarction.

Material and methods. The CRP precipitin test was performed according to the method of Anderson and McCarty(8). 1.5 cm each of CRP antiserum and the patient's serum were drawn up into a capillary tube (external diameter about 1 mm) and incubated for 2 hours at 37°C. The degree of precipitation (0 to 4+) was read after overnight refrigeration. Each millimeter of precipitate was considered 1+. Serial CRP tests were done on the sera of 5 of the 7 patients with coronary occlusion and myocardial infarction, and on the serum of one patient with acute coronary insufficiency and myocardial necrosis. Single specimens were tested in the other 2 patients with coronary occlusion. All these patients had a typical clinical course and showed the manifestations of necrosis, *i.e.,* fever, leucocytosis, elevated sedimentation rate, and elevated fibrinogen(11-13). These

TABLE I. CRP Tests in Patients with and without Myocardial Necrosis.

Sex	Age (yr)	Day of illness before first (+) test	Sedimentation rate (Wintrobe) mm/hr	CRP test
		Coronary occlusion; myocardial infarction		
♀	55	7	32	2+
♂	58	16	34	2+
♀	61	9	21	2+
♀	55	1½	42	1+
♀	53	3	30	3+
♂	52	2	20	3+
♂	54	2	30	2+
		Coronary insufficiency; myocardial necrosis		
♂	73	5	22	3+
		Coronary insufficiency without necrosis		
♂	59	2	8	Neg.
♂	58	7	5	"
♀	73	14	15	"
♂	50	7	30	"
♀	52	13	15	"
♂	41	7	20	"

* The CRP antiserum was kindly supplied by Schieffelin & Co. through the courtesy of Dr. E. W. Blanchard.

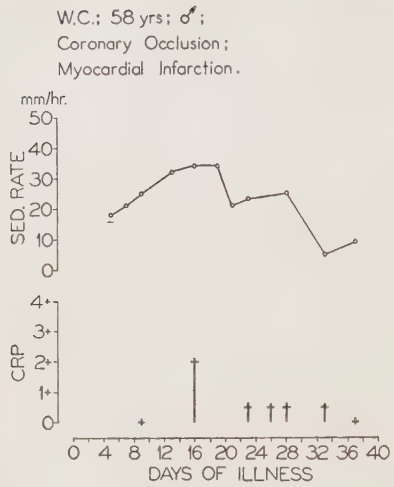

FIG. 1.

tests served as a positive control. Single CRP tests were done on the sera of 6 patients whose electrocardiograms showed RS-T and T wave changes characteristic of coronary insufficiency, but who had none of the manifestations of myocardial necrosis.

Results. Table I shows that the CRP test was positive in all 7 patients with myocardial infarction following coronary occlusion. The CRP was detected as early as 36 hours and was not detected as late as 5 to 9 days after onset of symptoms. It disappeared from the serum of one patient as early as the 15th day of illness. In 2 patients, minute amounts persisted during the 5th and 6 weeks of illness (Fig. 1). CRP was detected in the serum of one patient with severe coronary insufficiency and myocardial necrosis. His attack was precipitated by unusual effort, and the course was characterized by low-grade fever, elevated blood fibrinogen and sedimentation rate, and characteristic electrocardiographic changes.

In contradistinction, Table I shows that the CRP test was negative in 6 consecutive patients with coronary insufficiency but without signs of necrosis.

Discussion. Lofstrom(4), in his studies of pneumococcus C-polysaccharide, noted that sera of patients with myocardial infarction and fever formed a precipitate when the C-polysaccharide was added. Our data confirm this observation. The positive CRP test in all 7 patients with coronary occlusion and in the one patient with severe coronary insufficiency is unquestionably related to the presence of myocardial infarction and perinecrotic inflammation. The absence of CRP in the serum of some patients with acute myocardial infarction for as long as 5 to 9 days after the onset of illness (Fig. 1) suggests that a sufficient degree of inflammation is necessary for the elaboration of CRP. The extent of myocardial infarction is most probably the major factor contributing toward the degree of inflammation.

Coronary insufficiency(11) or coronary failure(12) may or may not be associated with myocardial infarction. RS-T and T wave changes characterize the usual subendocardial injury, but the injury may be reversible and not lead to infarction. Our data show that the CRP test was negative in patients with typical coronary insufficiency but without the criteria for myocardial infarction.

If these preliminary observations are confirmed by more extensive studies, the CRP test may prove to be a valuable aid in the differential diagnosis of coronary insufficiency, particularly as to the presence or absence of myocardial infarction. Furthermore, the pattern of the serial CRP tests in coronary occlusion with myocardial infarction (Fig. 1) suggests that the test may be useful in evaluating the subsidence of the inflammatory process in the heart.

Summary and conclusions. 1. Sera of 7 patients with coronary occlusion and myocardial infarction and of one patient with coronary insufficiency and myocardial necrosis were positive for CRP. 2. Sera of 6 patients with coronary insufficiency but without myocardial necrosis were negative for CRP. 3. These preliminary observations suggest that the CRP test may be a sensitive indicator of myocardial necrosis and inflammation.

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A Simplified Method for Determination of Lipide-C^{14} in Liver.* (21020)

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The present methods for determining lipide-C^{14} of tissues, in experiments in which slices are incubated with a C^{14}-labeled precursor, are time-consuming, and suffer from tedious manipulations, such as prolonged extraction and hydrolysis and repeated transferring of extracts from one vessel to another. These disadvantages have been completely eliminated in the simplified procedure described here. The entire analysis can be completed in less than 2 hours.

Procedure. The incubation flask is a 50-ml Erlenmeyer flask with a center well 10 mm in diameter and 20 mm high fused into the bottom. A self-sealing rubber cap is used as stopper. The general details of the incubation procedure have been described elsewhere(1). At the end of the incubation period, 0.25 ml of 30% KOH is introduced into the center well for absorption of CO_2 and, immediately thereafter, 0.25 ml of 5 N H_2SO_4 are added to the medium for inactivation of the tissue. Both alkali and acid are introduced by means of a syringe with a long hypodermic needle that is inserted through the rubber cap. Sufficient time (20-30 minutes at room temperature) is allowed for the absorption of CO_2 by the alkali, and the rubber cap is then removed. The contents of the center well are siphoned directly into a volumetric flask. The incubation medium is carefully filtered through Whatman No. 1 filter paper, so that the tissue slices are retained in the main compartment of the flask. The slices are thoroughly washed with several portions of distilled water, each of which is decanted onto the same filter paper. To avoid disintegration of the slices, the above procedure should be carried out no later than one hour after the reaction is stopped. The medium and washings are discarded unless a component of this fraction, such as glucose, ketone bodies, or the like, is to be analyzed. The filter paper is then washed with 5-10 ml of an alcohol-ether (3:1) mixture, and the washings are returned to the incubation flask. Two ml of 1 M sodium ethylate are then added to the tissue slices in the flask which is covered with a bubble

TABLE I. Test of Extraction Procedure.
To 500 mg ± 5 mg of liver slices was added one of the labeled compounds, and immediately thereafter the mixture was hydrolyzed. Extraction of the acidified hydrolysate was carried out as described in the text. Aliquots of the chloroform phase were directly mounted on aluminum discs and counted in the usual manner.

Labeled compound added to tissue slices	No. of exps.	% of added C^{14} recovered in chloroform phase (range)
*Fatty acid-C^{14}	6	99–101
Tripalmitin-1-C^{14}	3	98–100
Cholesterol-4-C^{14}	3	97–101
Alanine-1-C^{14}	3	0
Acetate-1-C^{14}	3	0
*Glycerol-C^{14}	6	0

* Biologically synthesized.

* This work was supported by a contract from the U. S. Atomic Energy Commission.

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stopper. The mixture is then hydrolyzed for 40 minutes on a steam bath. It is important not to allow the hydrolysate to become dry; if the volume becomes reduced during the heating, alcohol is added. At the end of this period, the bubble stopper is removed, 3 ml of water are added, and the heating is continued until the odor of alcohol is no longer detectable. The contents are then cooled, and acidified with 5 N H_2SO_4. Exactly 10 ml of chloroform are added from an automatic burette, an aluminum foil-covered stopper is inserted into the flask, which is shaken vigorously for about 2 minutes. The entire contents of the flask are then transferred to a 15-ml centrifuge tube. (This last transfer need not be quantitative. Since all lipides are now in the chloroform phase, measurements are made on aliquots of the original 10 ml of chloroform.) The tubes are centrifuged for about 5 minutes. Centrifugation separates the contents of the tube into an aqueous upper phase containing the water-soluble components and a lower chloroform phase containing the lipides. These 2 phases are separated by a thin plaque that is soluble in neither phase. Aliquots of the chloroform phase are removed by means of a syringe and a long hypodermic needle. For determination of lipide-C^{14}, a one-ml aliquot is mounted directly on an aluminum disk, and gentle heat is applied to evaporate the solvent. Aliquots of the chloroform phase may be withdrawn in a similar manner for determination of cholesterol or total fatty acids.

TABLE II. Test for Completeness of Hydrolysis. For each hydrolysis experiment, aliquots of an ether solution of tripalmitin or palmitic acid were delivered to duplicate flasks. Hydrolysis described in text. The mixture was acidified and cooled, and lipides extracted with chloroform as described in text. Aliquots of chloroform extract were evaporated to dryness in an atmosphere of CO_2, and fatty acid ester determinations were carried out in duplicate according to the method of Bauer and Hirsch (2).

Compound	Amt added, mg	Treatment	% recovery in $CHCl_3$ phase as fatty acid ester
Tr*	17	2 ml Na ethylate 30 min.†	0
Tr	17	4 ml Na ethylate 30 min.†	0
Tr	17	1 ml 90% KOH in 50% EtOH; 6 hr†	0
P	14	2 ml Na ethylate 30 min.†	0
Tr	17	0	100

* Tr = Tripalmitin; P = Palmitic acid.
† Time on steam bath.

TABLE III. Lipide-C^{14} Recoveries: A Comparison of Values Obtained by the Method Described Here with Those Obtained by Methods Used Earlier. 500 ± 5-mg liver slices were incubated in 5 ml bicarbonate buffer to which 2 μM acetate-1-C^{14} had been added. Incubation was carried out at 37.5° for 3 hr. Duplicate flasks were incubated, and their contents were analyzed separately; average values are reported below.

Rat	Labeled acetate added to flask, μmoles	% of added C^{14} recovered in lipides by: Method described here	Method of Chernick *et al.* (3)	Method of Felts *et al.* (1)
A	2	29		28
B	2	13		13
C*	2	2.8		2.8
D*	2	1.5		1.6
1M	10	4.5	4.4	
2M	10	4.8	4.9	

* Rats C and D were fasted for 48 hr.

Test of reliability of procedure. The reliability of the procedure was tested by the following experiments:

1. A biologically synthesized C^{14}-fatty acid mixture, cholesterol-4-C^{14}, tripalmitin-1-C^{14}, alanine-1-C^{14}, acetate-1-C^{14}, and glycerol-C^{14} were added to separate 500-mg portions of liver slices, and immediately thereafter the mixture was hydrolyzed and extracted with chloroform. The design and results of the experiment are given in Table I. Essentially all of the C^{14} of the added fatty acids, tripalmitin, and cholesterol was recovered in the chloroform fraction, whereas none of the C^{14} of the water-soluble compounds was present in that fraction.

2. The efficacy of the hydrolysis and extraction procedure was further determined with unlabeled tripalmitin and palmitic acid. The details and results of this experiment are shown in Table II. After each of the various periods of hydrolysis tested, none of the fatty acid ester was recovered in the chloroform phase. This shows that hydrolysis is complete under the conditions employed here.

3. The lipide-C^{14} recoveries observed with the method described here were compared with those obtained by methods used earlier in this laboratory, namely, that of Chernick *et al.*(3) and that of Felts *et al.*(1). The design and results of these experiments are given in Table III. It is clear that the lipide-C^{14} yields observed with the abbreviated method described here are in good agreement with those obtained by the laborious procedures employed earlier.

Summary. 1. A rapid method for determination of lipide-C^{14} in experiments in which liver slices are incubated with C^{14}-labeled compounds is described. 2. Data on the reliability of the procedure, and a comparison of values obtained by this simplified procedure with those obtained by the previous laborious methods are presented.

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Antigenicity of Canine Distemper Inclusion Bodies as Demonstrated By Fluorescent Antibody Technic. (21021)

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The fluorescent antibody technic was developed by Coons *et al.*(1) and is a method in which antibody conjugated to fluorescein is employed as a specific histochemical stain for the localization of antigen in cells. The technic has been used to demonstrate antigens of rickettsiae and mumps virus(1), pneumococcus(2,3), Friedländer bacillus(4), leptospira(5) and homologous plasma proteins in tissues(6). The method was recently employed to show viral antigen in the inclusion bodies of canine infectious hepatitis(7). This report deals with the application of the technic to cells containing inclusion bodies of canine distemper.

Materials and methods. Fluorescein isocyanate was prepared and conjugated to protein of anti-canine distemper serum* according to the method of Coons and Kaplan(8). Absorption of the conjugate on liver powder for preventing non-specific fluorescence in tissues was not found necessary in the smear preparations which were used. Smears of urinary bladder epithelium from natural cases of canine distemper were used as a source of inclusion bodies. Smears were made on coverslips which were subsequently dried 30 minutes before staining. The staining procedure was as follows: A smear was treated for 20 minutes with the specific conjugate and the excess shaken off. The smear was washed for a few seconds in buffered saline, pH 9, and then gently agitated in fresh buffered saline for 10 minutes. The coverslip was dried and mounted smear surface down over a square aperture in a slide. *Fluorescence* in the smears was produced with ultraviolet light and observed through a standard microscope. The light source was a Leitz, 8 ampere, carbon arc. Filters consisted of 3.2 cm of $CuSO_4$ (25 g/100 ml) in a pyrex cuvette and Corning filter No. 5840 (½ standard thickness) to remove visible light. The filtered ultraviolet radiation practically free of visible light was directed into an ordinary glass substage condensor by means of a polished mirror of aluminum-magnesium alloy. A protecting filter (Wratten gelatin filter No. 2A) was mounted in the ocular of the microscope. Photomicrographs of fluorescence were taken with Eastman Super Panchro-Press, Type B film with 20-minute exposures. After a suitable area of fluorescence was located in the smear and photographed, the slide was re-

* Obtained through courtesy of Dr. Hilary Koprowski, Lederle Laboratories.

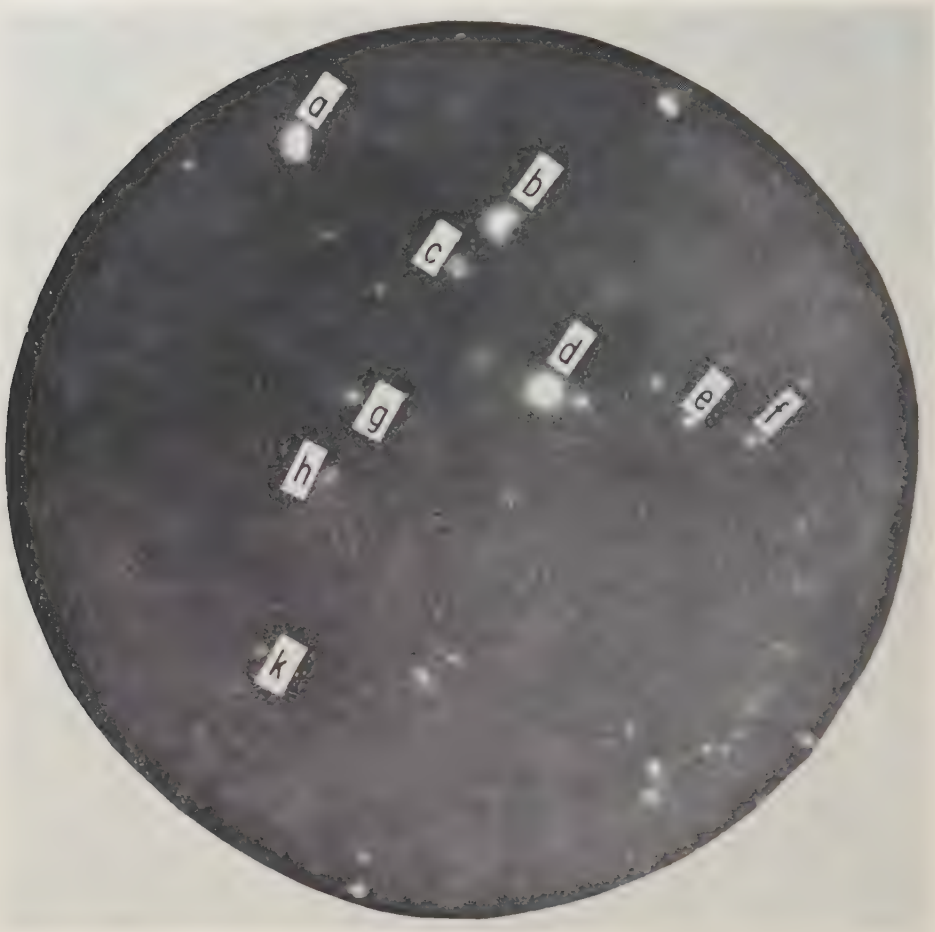

FIG. 1a

moved and stained with Sellers' stain(9). The slide was then replaced in the microscope and a photograph of the same field was taken with visible light. In this manner by comparing photographs, fluorescent objects could be correlated with their color-stained counterparts. *Specificity of staining* was determined by 1) treating bladder smears from infected dogs with normal distemper antiserum for 10-20 minutes prior to staining with conjugate and 2) by staining smears from normal dogs with conjugate.

Results. Under ultraviolet light widely scattered, green fluorescing deposits were observed in the smears. Photographs of these fluorescent structures compared with photographs of the same structures stained with Sellers' stain showed that fluorescence was

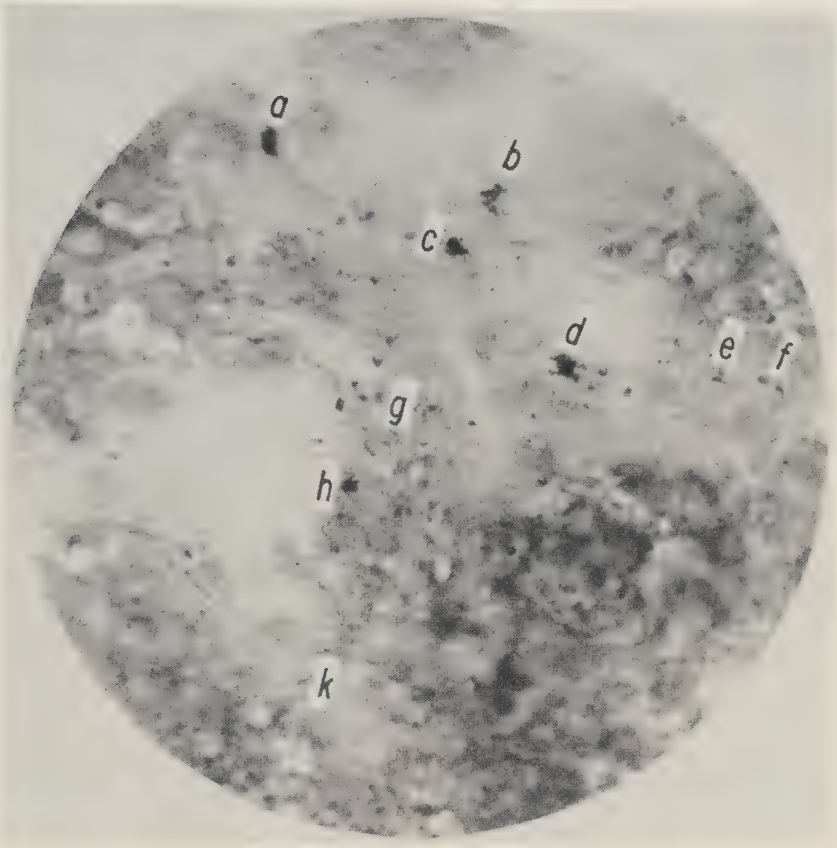

FIG. 1b

FIG. 1. Smear of urinary bladder for comparison of inclusion bodies (a) treated with fluorescent antibody and (b) stained by Sellers' method. Fields are identical. Letters point out corresponding inclusion bodies. × 300.

localized in the inclusion bodies of distemper (Fig. 1). Some distortion was noted in the inclusion bodies after the final staining, but this distortion was not observed in fresh smears of the same material stained immediately with Sellers' (Fig. 2).

When smears containing inclusion bodies were treated with normal antiserum before staining with conjugate, fluorescence was partially inhibited. Smears of normal canine bladder epithelium showed no specific fluorescence when stained with conjugate.

Summary and conclusion. Canine distemper inclusion bodies in urinary bladder epithelium were specifically stained with fluorescein-antibody conjugate. On the basis of this finding it is concluded that these inclusion bodies contain viral antigen.

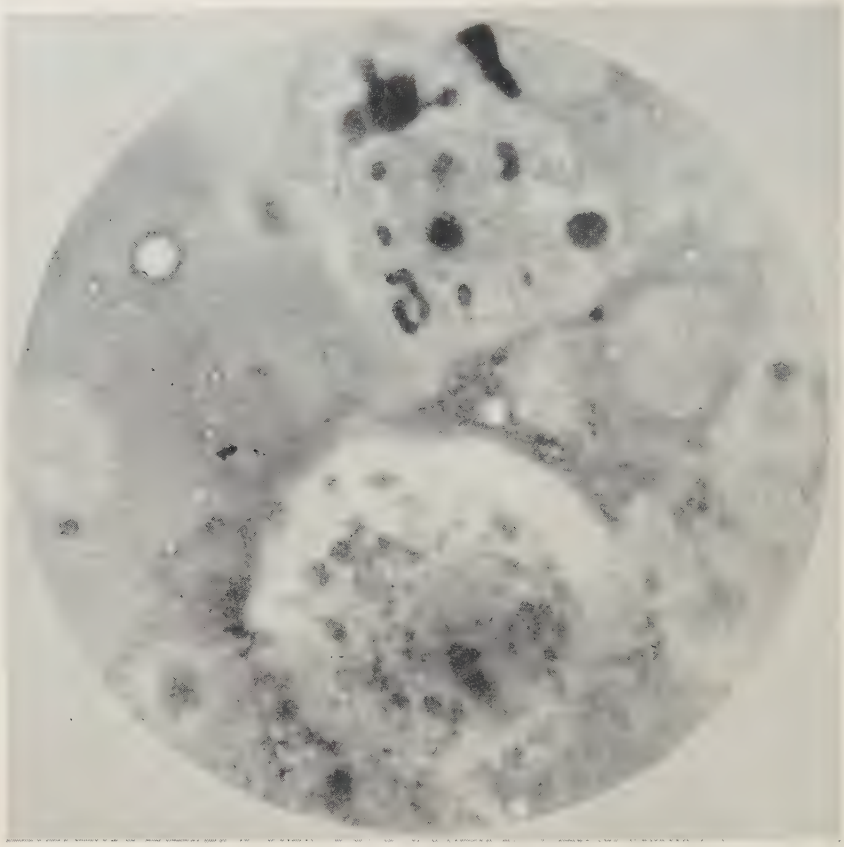

FIG. 2. Fresh smear from same urinary bladder as in Fig. 1 showing epithelial cells filled with inclusion bodies. Sellers' stain. × 900.

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Rate of Respiration of Tissues in Contact with Oxygen.* (21022)

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Standard Warburg methods for the *in vitro* study of tissues involve suspension of the tissue slices, or of various types of tissue homogenates, in volumes of suspension fluid greatly in excess of the volume of the tissues. Many different solutions have been used with the aim of approximating the *milieu intérieur* so that the rates of respiration obtained might represent those in the intact animal. Quite different values are obtained with different solutions and it is difficult to know which, if any, represents the *in vivo* condition. Since suspension of tissues in a liquid results in dilution of metabolites to a level below the optimal extracellular fluid level, Krebs(1) has proposed a medium which makes a partial restitution of these metabolites. However, this solution does not compensate for dilution of hormones normally present or other of the many materials to be found in the cells of living tissues. On the other hand, the added material might stimulate the rate of tissue respiration beyond the normal *in vivo* rate. The uncertainties of the physiologic picture complicate the interpretation of *in vitro* pharmacologic investigations. Furthermore, a drug in a quantity which may produce a significant effect on the intact animal may be so diluted when the tissue is placed in a Warburg vessel that little or no effect can be observed. To demonstrate cellular effects the investigator must often resort to the addition to the suspension medium *in vitro* of forms and quantities of material it is hoped will duplicate conditions in the intact animal. There would appear to be need for a method for the quantitative assessment *in vitro* of the tissue actions of a drug administered *in vivo*.

This paper presents a method that meets this requirement by the use of specially-designed vessels in which the tissues are carefully spread out on fiber-glass mats to permit ready contact of gas with both sides of the slice. Such a method is so different from standard technics as to require critical evaluation. One test of the validity of a method of measuring tissue respiration is a comparison of the summated value with the BMR. Field, *et al.*(2) carried out a summation of tissue metabolism in the white rat which accounted for about 66% of the BMR. They felt this result indicated that the rates of tissue respiration *in vitro* bore a reasonable relationship to the rates *in vivo*. In the work that follows we have compared the respiratory rate of slices from the same organ on mats, in Krebs-Ringer-Phosphate (KRP) solution and in Krebs Medium III (KMIII)(1); and have assessed the significance of the results by summation calculations.

Methods. 1. *Apparatus.* When an attempt is made to study tissues directly in a gas phase it is difficult to assure adequate diffusion of gas to the under surface of the tissue slice. If a slice were to be cut thin enough to allow an adequate supply of oxygen from one side only there would be too high a proportion of damaged cells and the slice would be very difficult to handle. A solution to the problem is found by the use of an inert glass-fiber mat which supports the tissue effectively yet permits free access of gas to the under surface. Such a material is available from battery manufacturers under the name of "battery mat". This product is a feltwork about 0.5 mm thick of fine glass fibers bonded together with a small amount of starch. The starch may be removed by treatment with acid but we found no difference in respiratory rate after such treatment and the starch lends useful stiffness to the mats. Thin slices of tissue are spread out without folds or overlapping on tared ovals of mat cut with scissors to suitable size. Thereafter the tissues need not be touched. Such a mat and tissue may be forced into an ordinary Warburg vessel, as was done in preliminary experiments in this

* Supported by funds for research in biology and medicine under Initiative Measure 171 of the State of Washington.

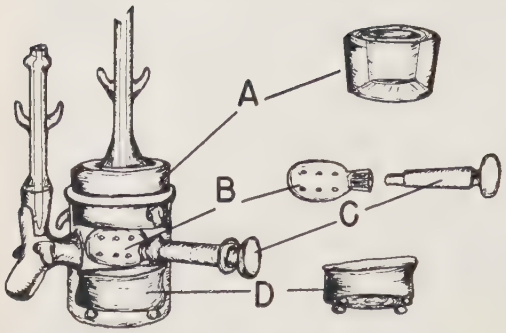

FIG. 1. A. Adapter to attach vessel to standard manometer. B. Paddle. C. Handle of paddle. D. Removable tray.

study. However, since it is practically impossible to keep the tissue spread evenly as is necessary for optimal respiratory exchange, a wide-mouthed vessel† was designed and is briefly described here (Fig. 1). The vessel, of about 19-ml capacity, is attached to a standard manometer by a glass adapter. A removable tray (D) with 4 small legs sets on the bottom of the vessel. A mat bearing the tissue is placed on a rotatable paddle (B) above the tray. When used for studying oxygen uptake, KOH solution is placed on filter paper on the bottom of the vessel and Ringer or other solution is placed in the tray to maintain normal vapor pressure. The apparatus can be used for standard procedure by placing the tissue not on the paddle but in the solution in the tray. The paddle was made rotatable so that the mat and tissue might be dropped into the solution in the tray. This is desirable for 2 types of experiment: (a) for measurement of the R.Q. Here the tissue is dropped into strong acid in the tray and the carbon dioxide which is released is absorbed by alkali formed by the interaction of KI solution from the side arm and $KMnO_4$ solution on the bottom of the flask, after the second method of Dickens and Simer(3); (b) to test the effect of dilution on drug action. Here the effect of a drug administered *in vivo* is determined with the tissue on the paddle and, after an interval, the tissue is dropped into a solution in the tray and the experiment continued but now with shaking. Significant dilution and diffusion of the drug would be detected by changes in the rate of oxygen consumption.

2. *Procedure.* The animals used were male albino rats averaging 263 g body weight. The animals were killed by crushing the cervical vertebrae or exsanguination by heart puncture. The organs to be investigated were immediately removed in a moist cold chamber (0-5°C). Skeletal muscle, except diaphragm, was prepared by teasing out small groups of fibers. Diaphragm was excised *in toto* and pieces cut parallel to the fibers with scissors. Liver, kidney, and spleen were cut with a Martin slicer(4); brain and heart were cut through a template(5) with a razor blade. Pieces of intestine, including both mucosa and muscle layers, were taken from the small intestine beginning about 6 inches from the pylorus. The testes were cut with scissors. The tissues were spread out carefully on the fiber-glass mats, weighed and kept in moist Petri dishes in the cold chamber until all had been weighed. The mats were then placed on the paddles of the vessels which had been warmed to 37°C on a hot plate. Of each organ studied usually 2 samples were placed on mats, 2 in KRP and 2 in KMIII. The tissues not on mats were weighed on tared pieces of waxed paper and removed to the solution in the tray. The oxygen consumption was determined by the direct method of Warburg using a gas phase of oxygen. The determinations were carried out at a temperature of 37.9°C and at a shaking rate of 125 cycles per minute whenever tissues were suspended in solution in the tray, which normally received 1.5 ml of solution. CO_2 was absorbed by 0.2 ml of 5% KOH on a piece of filter paper on the bottom of the flask. The equilibration period in the water bath was approximately 15 minutes during which period it proved essential that all ground glass connections be thoroughly tightened. Readings were taken at 10-minute intervals for the first hour and at 20-minute intervals thereafter.

Results. QO_2. The rate of tissue respiration in artificial media usually declines to some extent with time. Since the tissues we used were kept in a cold chamber until all were ready to be placed in the vessels, the respiratory rate would be low until the vessels

† Obtained from E. Machlett and Co., New York City.

TABLE I. Metabolic Levels *In Vitro*. Figures represent mean QO_2 values in ml O_2 per g wet wt per hr at time of setting and at 60 min. thereafter.

Organ	On mats					In Krebs-Ringer-Phosphate					In Krebs Medium III				
	No. of exps.	0′	s.d.	60′	s.d.	No. of exps.	0′	s.d.	60′	s.d.	No. of exps.	0′	s.d.	60′	s.d.
Brain	35	2.77	.42	1.24	.27	12	1.39	.35	1.04	.14	13	2.21	.56	1.92	.35
Heart	24	2.86	.45	1.82	.43	6	1.55	.17	.94	.34	12	1.81	.34	.85	.37
Intestine	20	1.38	.41	.92	.28	6	1.08	.26	.65	.18	12	1.35	.35	1.13	.24
Kidney	22	4.68	.64	4.08	.47	5	3.72	.49	3.54	.28	10	2.76	.58	2.75	.15
Liver	32	3.06	.48	1.95	.36	5	1.70	.20	1.59	.32	6	3.11	.36	2.43	.17
Skeletal muscle															
Sterno-hyoid, sterno-mastoid, sterno-thyroid	24	1.33	.29	.83	.23	7	.89	.19	.55	.17	15	.92	.19	.68	.15
Ext. oblique, int. oblique, rectus abd.	17	1.01	.22	.57	.26										
Caudofemoralis and semitendinosus	12	.98	.18	.49	.35										
Diaphragm	26	1.62	.32	1.42	.21	14	1.18	.17	1.18	.10	13	1.42	.15	1.34	.15
Spleen	25	1.56	.25	1.46	.15	19	1.34	.13	1.34	.13	8	1.85	.03	1.79	.12
Testis	27	.81	.09	.51	.01	6	.64	.08	.52	.07	14	.89	.16	.87	.19

and tissues were warmed to body temperature by the water bath. The tissues would be expected to return to their maximal respiratory rates at the conclusion of equilibration. The respiratory rates at this time were determined by straight line extrapolation of rates obtained during the experimental period. The results obtained with 9 tissues are recorded in Table I. So that it may be possible to compare rates of decline of tissues studied by the 3 methods we have included in the table the rates of respiration at 60 minutes.

A striking feature of the findings of this investigation is the remarkably high respiratory rates of many tissues on mats. These values are so much higher than those of equivalent tissues suspended in KRP as scarcely to require statistical examination. In comparison with tissues in KMIII the application of Fisher's "t" test demonstrates the following: (a) at zero time on the mats the rates are reliably higher for muscle, brain, kidney, and heart; the rates are the same for liver, intestine and testis; and lower only for spleen. (b) At 60 minutes on the mats the rates are reliably higher for muscle ($p = 0.05$), kidney and heart; and the rates are reliably lower for liver ($p = 0.04$), intestine ($p = 0.05$), brain, testis, and spleen. While the rate of decline of the tissues on mats is not excessive it is apparent that it is somewhat more marked than in KMIII.

pH. When living tissues are studied *in vitro* there is always a possibility of changes in pH. A series of experiments was therefore performed with brain, intestine, kidney, liver, and muscle in which the pH of each tissue was followed during the experiment. Serum exposed to the low CO_2 tension characteristic of the direct Warburg method progressively loses CO_2 to the KOH in the vessel and becomes strongly alkaline. Even though some serum and extracellular fluid accompany the cells of the tissue slice what changes took place were in the acid direction. Muscle and brain showed essentially no change in pH. Intestine and kidney showed a very slight drop, and liver showed the most change with a drop of approximately 1.5 pH units after one hour. The production of acid material by the respiring tissue was noted also when tis-

sues were run in fluid media. When control experiments were undertaken on the media in which no tissue was suspended, the well-buffered KRP showed little change of pH but the weakly-buffered KMIII became more alkaline. When tissues were suspended in the latter medium the pH increased slightly but did not become as alkaline as the controls. Since it has been shown by Canzanelli *et al.* (6) that the respiration rates of tissue slices are influenced by changes of pH it may be that a part of the higher values obtained with KMIII with some tissues is due to an increase in pH.

Summated tissue respiration. In order to apply summated tissue respiration as a criterion of the validity of these 3 modifications of the Warburg technic certain essential computations must be performed. We have followed the same general procedures as Field *et al.*(2); and for those tissues we have not reinvestigated, which account for less than 15% of the total summated respiration, have used their values of QO_2. The mean starved weight of the animals used was 263 g. The weights of the several organs were calculated after Donaldson(7), and Field *et al.*(2). In the case of skeletal muscle the figure of 45.4% of the body weight was used. Since it has been shown(8) that in the rat about 3.4% of the striated muscle consists of relatively inert connective tissue, the weight of skeletal muscle was reduced by this amount. Diaphragm, however, was not corrected for connective tissue. The BMR was calculated from Davis (9) using the values obtained for the sleeping animal. For a 263 g rat the oxygen consumption is 229.3 ml per hour. The QO_2 value used for skeletal muscle on mats is the unweighted mean of the values for neck, abdominal and leg muscles. In the case of skeletal muscle in KRP and KMIII only neck muscles were used. The figure for intestine was used for the entire alimentary canal.

The results of the tissue summations are contained in Table II. The total by our technic is 233.4 ml of oxygen per hour which is 101.8% of the BMR. The value in KRP is 180.4 ml per hour which is 78.7% of the BMR; and in KMIII is 207.9 ml per hour or 90.7% of the BMR.

Discussion. The technic of determining tissue respiration with the tissues on mats in an atmosphere of oxygen would appear to yield reproducible values of general physiologic importance. The observation that the respiratory rate of tissues can be measured without the introduction of the uncertainties of an artificial liquid medium, opens up the possibility of physiologic and pharmacologic investigations not previously practicable.

It is our feeling that the results obtained by the new method may, in many cases, be taken to be the most reliable approximation to *in vivo* conditions yet available since there is a minimal adjustment necessary to a foreign environment. No substances are added and none are lost excepting only CO_2 and some water. The O_2 tension is the same as that employed in the other usual technics. On the positive side there is evidence that the tissues are in a physiologic state. The pH changes are small and in the direction of tissue activity. The decline in respiratory rate is gradual and only slightly more marked than in the best standard technics. Despite the necessity for making certain assumptions the results of the tissue summation calculations are gratifying. Using KRP it was possible to account for 79% of the BMR which is somewhat higher than that reported by Field *et al.*(2). As might be expected the fortified solution proposed by Krebs(1) yielded a higher total which accounted for 91% of the BMR. This appears to us a rather satisfactory equivalency and lends support to the further use of this medium in experimental procedures where it is desirable to have the tissues suspended in a fluid medium. The summated tissue respiration calculated from the values on mats is the highest at 102% of the BMR and, to our minds, helps to complete the proof that the respiratory rate of tissues measured *in vitro* is of the same order of magnitude as when functioning in the intact animal. We do not feel that it is a cause for concern that the value comes to more than 100% of the BMR in view of the estimates necessary in the calculations and the uncertainty of methods of determining the proportion of metabolically active tissue in an organ (for a discussion see Martin and Fuhrman

TABLE II. Summated Tissue Respiration in the Rat.

Organ	Organ wet wt in 263 g rat	Field, *et al.*(2) QO_2	Mean O_2 consumption at time of setting manometers — On mats QO_2	On mats Whole organ	In Krebs-Ringer-Phosphate QO_2	In Krebs-Ringer-Phosphate Whole organ	In Krebs Medium III QO_2	In Krebs Medium III Whole organ
Alimentary canal	12.2		1.38	16.8	1.08	13.2	1.35	16.5
Brain	1.9		2.77	5.1	1.39	2.6	2.21	4.2
Heart	1.0		2.86	2.9	1.55	1.6	1.81	1.8
Kidney	2.2		4.68	10.3	3.72	8.2	3.76	8.3
Liver	12.4		3.06	38.0	1.70	21.1	3.11	38.6
Skeletal muscle	115.3		1.11	128.0	.89	102.7	.92	106.1
Diaphragm	1.7		1.62	2.8	1.18	2.0	1.42	2.4
Spleen	.7		1.56	1.1	1.34	.9	1.85	1.3
Testis	2.5		.81	2.0	.64	1.6	.89	2.2
Blood	15.3	.025		.4		.4		.4
Integument	47.3	.362		17.1		17.1		17.1
Ligaments	13.0	.070		.9		.9		.9
Lungs	1.5	1.15		1.7		1.7		1.7
Skeleton	15.5	.145		2.3		2.3		2.3
Remainder	20.5	.200		4.1		4.1		4.1
Totals	263.0			233.5		180.4		207.9
% of BMR	(229.3 ml/hr)			101.8%		78.7%		90.7%

(10)). The fact that the tissues on mats metabolize at a high rate may be due to more adequate oxygenation and preservation of catalytic substances at all stages of essential metabolic cycles.

It is not surprising that the rate of decline of brain respiration on mats is relatively marked in view of the well known ability of this tissue to exhaust its supply of carbohydrate rapidly. We therefore feel that the high initial value is sound and will be substantiated by other work.

Many investigators in the field of tissue respiration would agree with the comments of Bertalanffy and Pirozynski(11) in their concern over variations introduced by different media amounting to 2- to 3-fold. It may be that our technic offers a solution to some of these problems.

Summary. 1. A method is described for the determination of the respiratory rates of tissues in contact with oxygen by supporting them in the gas phase on fiber-glass mats in a modified wide-mouthed Warburg flask. 2. Investigated in this way the tissues maintained a satisfactory physiologic state as evidenced by slow decline in respiratory rate and negligible changes in pH. 3. For 8 rat tissues studied the respiratory rates on mats were considerably higher than those in Krebs-Ringer-Phosphate; and higher than those in Krebs Medium III in the case of muscle, brain, kidney, and heart, lower only for spleen, and not significantly different for liver, intestine, and testis. 4. Application of the respiratory rates to summated tissue calculations yielded the following values relative to BMR: on mats, 101.8%; in Krebs-Ringer-Phosphate, 78.7%; and in Krebs Medium III, 90.7%. 5. The utility of the technic using mats as a means of avoiding the variables due to different liquid media is discussed.

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Urinary Free Amino Acid Excretions During Successive Pregnancies. (21023)

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(Introduced by I. M. Hoobler.)

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Investigators(1-9) have shown that there is little correlation between the quality or quantity of protein ingested and the amounts of amino acids excreted. Wallraff(10) reported that the free, rather than the total amino acids are excreted in significantly higher amounts during pregnancy. A study in this laboratory of 15 women during the reproductive cycle(11) disclosed that the excretion of specific free amino acids may be used as an index of physiological adaptation of the maternal body to the growth and development of the products of conception. In the investigation reported here, 24-hour collections of urine from one woman during 3 successive reproductive cycles were analyzed for free threonine, histidine, lysine, tryptophan, phenylalanine, isoleucine, valine, leucine, arginine, and methionine.

Experimental. The subject of study is a white American woman 5 feet, 7 inches tall and weighing approximately 150 lb, who was studied while she was 23 to 25 years old. Her blood type is B, Rh negative. Before pregnancy she had a basal metabolic rate of −23 and between the second and third pregnancies, the rate was +1. Frequent medical examinations before and throughout the study showed her to be in good physical health. For the subject, ranges of weight, blood pressure, hemoglobin, red and white blood counts during the 30-month study period are given in Table I. Estimated length of gestation and the infants' birth weights also are included. Carefully-checked dietary history and food intakes recorded for each 24-hour period in which urine samples were collected indicated that the subject's dietary habits were good. Eighteen 24-hour urine samples were collected for amino acid analysis. One sample was obtained when the woman was nulligravid; 8 samples were collected at approximately monthly intervals during her first pregnancy, the first in the second month (44 days after the last menstrual period), and a sample was procured during lactation, 47 days postpartum; 6 samples were obtained during the second reproductive cycle, one 6 months postpartum when the subject was not lactating; and during the third pregnancy, urine samples were collected in the second and fourth months. All samples were preserved with small amounts of glacial acetic acid stored at −20°C pending analysis. Microbiological methods reported previously(12) were used in determining the 10 free amino acids. The organisms used were *Lactobacillus plantarum* for leucine, isoleucine, and valine; *Leuconostoc mesenteroides* for histidine, lysine, methionine, and phenylalanine; and *Streptococcus faecalis* for arginine, threonine, and tryptophan. Total assay volume was 3 ml and growth responses were measured turbidimetrically. Amino acid values reported are averages of 2 or more assays in which determinations were run in duplicate at 5 levels. Duplicate assays which did not check with ±5% were repeated.

Results. The quantities of free amino acids excreted by the subject in the 24-hour collections of urine obtained during the 3 reproductive cycles are given in Table II.

Compared to nulligravid values for the subject, gravida I excretion of each of the 10 amino acids was augmented. Increases are evident from levels in the second month for all of the amino acids except methionine. Decreases in levels of excretion for each amino acid occurred in the fourth or fifth month except for threonine and methionine. From the sixth to the ninth months, excretion levels of most of the amino acids increased slightly or remained fairly constant. Excretion of threonine continued to increase as gestation progressed, while that of methionine fluctuated slightly from the nulligravid value.

TABLE I. Clinical Observations during Consecutive Pregnancies.

	1st pregnancy*	2nd pregnancy*	3rd pregnancy*
Wt, lb	150-173	149-165	149-157
Hemoglobin, g/100 ml	11.5 (7th mo)	14.2 (7th mo)	13.9
Red cell count	4.16(")	4.60(")	4.62
White cell count	8900 (8th mo)		8100
Blood pressure	100/60 to 128/70	100/60	110/70 to 124/60

* First pregnancy resulted in birth at term of a living male weighing 7 lb 9 oz; second pregnancy concluded by birth of a living premature male infant (7 mo), weighing 3 lb 12 oz; third pregnancy has not terminated.

When gravida II, the subject's excretions showed a pattern similar to that of her first pregnancy, but the levels of excretion of most of the amino acids were higher in the second month and lower in the third and fourth months. Of the 10 amino acids determined, only the excretion of threonine and leucine were as high during the second as during the first pregnancy. The second pregnancy terminated after 7 months. While trends of amino acid excretion were similar in the first two pregnancies, the quantities excreted in relation to duration of gestation were different.

The two samples collected and analyzed when the mother was gravida III showed increased amino acid excretions early in gestation similar to levels in the 2 preceding pregnancies.

The 10 amino acids were excreted during lactation in quantities not only smaller than during gestation, but also less than those found in the nulligravid state. Threonine, histidine, lysine, and tryptophan which during pregnancy showed the greatest increases in excretion over the nulligravid state also showed the greatest decreases in lactation.

In the postpartum urine sample obtained from the subject when not lactating, quantities of threonine, histidine, lysine, and phenylalanine were similar to those found when she was nulligravid. The other six amino acids however, were being excreted at levels lower than when she was nulligravid and approximating those found during lactation.

Discussion. The excretion of individual amino acids is affected differently by the metabolic alterations in response to the increased nutritive demands of reproduction. Of the 10 amino acids studied, the excretions of threonine, histidine, lysine, and tryptophan were most affected. Fig. 1 illustrates for the 4 amino acids, the relationships at different intervals in the reproductive cycle between levels of excretion and the initial non-pregnant values. Only threonine showed an increasing ratio of excretion throughout gestation which reached maxima of 11- and 12-fold in the first and second pregnancies, respectively. Histidine, lysine and tryptophan reached maximum ratios of 5-, 4- and 3-fold increases, respectively, during the first trimester. In general, during the first pregnancy amino acids in the urine tended to decrease during the second and increase during the third trimester; in the second pregnancy which terminated prematurely, the increase occurred in the fifth and sixth months. Leucine, isoleucine, valine, methionine, phenylalanine and arginine showed patterns similar to that of lysine, but the changes in their excretion only varied about 2-fold during the entire reproductive period.

Biochemical changes and physiological adaptation of the maternal body are known to be initiated as early as the second month in gestation and are demonstrated by increased urinary excretion of the 10 free indispensable amino acids(11). The products of conception also are alleged to alter the hormonal balance(13); change the basal metabolic rate of 13% or more in excess of that conditioned by the gross increase in body weight(14); modify the distribution of plasma proteins(15); and vary the concentrations of hemoglobin, sera vit. C, vit. A, carotenoids, and alkaline phosphatase(16)—all of these changes occurring to accommodate the maternal organs and tissues to the increased nutritive needs and functions necessary for the growth of the mother and fetus(17). The maternal dietary requirements for protein, minerals and vitamins also are increased by

TABLE II. 24-Hour Urinary Excretion of Free Amino Acids in mg during the Reproductive Cycle.

Amino acid	Gravidity	Pregravid	Month of gestation 2	3	4	5	6	7	8	9	Postpartum Lactating	Non-lactating
Threonine	0	18.3										
	1		39.3	118	119	163	156	180	199	187	14.5	
	2		49.3	78.8	88.1	201	218					22.4
	3		30.9		74.2							
Histidine	0	115										
	1		291	570	627	581	548	556	527	552	68.9	
	2		349	520	415	494	572					112
	3		340		457							
Lysine	0	55.1										
	1		131	249	183	217	167	168	212	204	21.8	
	2		126	192	125	157	173					64.2
	3		165		186							
Tryptophan	0	15.1										
	1		29.7	50.3	44.7	43.4	41.5	48.6	53.5	45.2	6.1	
	2		32.4	40.1	32.2	44.9	48.2					11.8
	3		24.2		30.8							
Phenyl-alanine	0	10.8										
	1		16.1	17.0	22.0	21.3	19.4	18.8	21.8	16.1	4.4	
	2		19.5	18.1	12.1	14.8	19.0					5.2
	3		14.9		14.9							
Isoleucine	0	6.7										
	1		9.3	16.5	11.3	10.5	8.6	8.0	11.1	10.5	4.6	
	2		8.8	7.7	5.5	7.2	7.4					3.9
	3		6.2		5.5							
Valine	0	8.5										
	1		11.2	18.2	13.5	13.7	12.7	12.0	14.8	14.5	6.3	
	2		15.6	17.8	10.1	10.0	14.2					4.9
	3		10.6		10.4							
Leucine	0	11.1										
	1		14.5	25.8	20.0	23.4	17.5	15.6	20.7	23.0	8.3	
	2		27.8	16.4	10.1	10.4	13.6					4.8
	3		12.5		13.0							
Arginine	0	8.4										
	1		9.7	10.2	10.9	10.2	8.5	9.0	9.7	10.2	4.4	
	2		9.8	9.6	5.6		9.4					5.0
	3		8.5		9.4							
Methionine	0	3.1										
	1		2.7	3.5	3.8	3.8	3.8	3.0	3.7	2.8	1.1	
	2		3.3	1.6	1.4	2.3	3.4					1.2
	3		2.6		2.5							

needs for the metabolic and physiologic functioning of the developing fetus and for the nutritional conditioning(18), or reconditioning (19-21), of the maternal body in preparation for labor, parturition, and lactation.

The changed levels in urinary excretion of free amino acids during pregnancy is one of many manifestations of biochemical transformations that accompany physiological stress(22) and increased metabolism. Only for free threonine does increased urinary excretion persist throughout gestation, the other 9 amino acids seem to be conserved after the third month of pregnancy. The physiological economy of the maternal body during the reproductive cycle is one of intricate biochemical balance in the coordination of the physiological functions and demands of the maternal, fetal, and placental structures in sharing the nutrients available from the maternal diet. The decrease in amino acid excretion during lactation may be the result of the increased demand by the mammary glands for amino acids to synthesize protein for milk. This

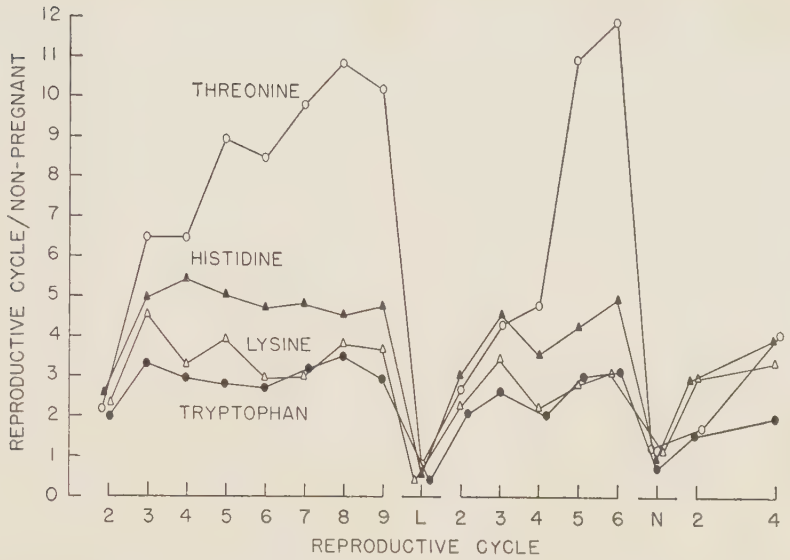

FIG. 1. Comparison of intensities of change of excretory levels of 4 amino acids based upon ratio of periods during reproductive cycle to the non-gravid state. Reproductive cycle is designated by numbers of months of gestation: L, lactating period; N, non-pregnant, postpartum gravida II period.

decrease may also be an effort on the part of the body to replace the amino acids lost during parturition.

Present methods for studying healthy mothers during pregnancy do not reveal what proportion of the maternal urinary excretion of free amino acids are contributed through the metabolic activity of the placenta, the fetus, or the mother. The growth rates of the placenta and fetus do not parallel one another(23). The metabolic activity of the embryonic protoplasmic mass increases throughout interuterine life, whereas the oxygen consumption of the placenta has been shown to decrease as early as the tenth or twelfth week of gestation(24), or as soon as the fetal organs begin to function. The organogenesis of the human fetus is completed during the first trimester of pregnancy. The early alteration in the distribution of maternal amino acid excretion during gestation therefore, coincides with a rise in maternal metabolism, an increase in active placental and embryonic protoplasmic mass, stimulated hormonal activity, and fetal kidney function.

The excretion patterns for each amino acid during each of the three reproductive cycles for this woman were similar in trend to the pattern established by the median values in the cross-sectional study of 15 women.

While amino acid patterns during pregnancy are similar to and coincide with many physiologic changes, there is need for study of more subjects to evaluate the biochemical changes in reproduction and lactation.

Summary. 1. Eighteen 24-hour urine samples collected from one woman during nulligravid, gravid and lactating periods, were analyzed for the free indispensable amino acids—leucine, isoleucine, valine, histidine, lysine, methionine, phenylalanine, arginine, threonine and tryptophan. The excretion level for each amino acid increased from the nulligravid to the gravid state, evident as early as the second month of gestation. Excretion of threonine continued to increase during gestation while methionine fluctuated slightly from the non-pregnant value. The other 8 amino acids showed a decreased excretory level during the fourth and fifth months of gestation. From the sixth to the ninth months, excretion levels of most of the amino acids increased slightly or remained fairly constant. 2. During lactation, the excretion level for each amino acid is less than

that for pregnancy or non-pregnancy. 3. Threonine, histidine, lysine and tryptophan showed the greatest changes from one phase of the reproductive cycle to another. 4. While trends of amino acid excretion were similar from one pregnancy to another, the quantities excreted in relation to duration of gestation were different.

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Production of Protective Antigens by *Pasteurella pestis* in a Synthetic Medium. (21024)

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(Introduced by Riley D. Housewright.)

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Wright *et al.*(1) have reported the production of immunogenic substances by *Bacillus anthracis* in a synthetic medium. It is the purpose of this note to report preliminary observations of a similar nature with *Pasteurella pestis.*

Materials and methods. The avirulent strain A-12 of *P. pestis,* a single colony isolate of strain A-1122 of Jawetz and Meyer(2), was cultivated in a synthetic medium based on that developed by one of us (K.H.). The formula for the medium is given in Table I. The amino acids were dissolved in the salt solution by heating and, after cooling, the mixture was adjusted to pH 7.5 with 10 N NaOH. The medium was distributed in 234 ml amounts into 2.8 l Fernbach flasks and

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TABLE I. Formula for Synthetic Medium Used for Production of Immunogenic Substances by *Pasteurella pestis*, Strain A-12.

Ingredient	g/liter	Ingredient	g/liter
l glutamic acid	12.0	K_2HPO_4	2.2
dl phenylalanine	.8	KH_2PO_4	1.7
dl methionine	.48	$MgSO_4 \cdot 7\ H_2O$	4.9
dl valine	1.60	Na gluconate	2.2
dl leucine	.4	$FeSO_4 \cdot 7\ H_2O$	.0028
dl lysine HCl	.4	$MnSO_4 \cdot H_2O$	.0017
l proline	.8	$NaHCO_3$†	2.5
dl threonine	.32	thiamine HCl‡	.001
l cysteine HCl	.1	Ca pantothenate‡	.001
glycine	.1	biotin‡	.0005
dl isoleucine	.62	distilled water	1 liter
xylose*	5.		

* Prepared as a 25% solution and sterilized at 121°C for 15 min. Added to the medium before inoculation.

† Prepared as a 6.25% solution and sterilized by filtration through a sintered glass filter. Added to the medium before inoculation.

‡ Prepared as a stock solution containing 0.25 mg/ml each of thiamine HCl and Ca pantothenate and 0.125 mg/ml of biotin. Sterilized by filtration through a sintered glass filter. Added to the medium before use.

autoclaved at 121°C for 15 minutes. Immediately before use xylose, the solution of vitamins, and sodium bicarbonate were added aseptically. A 24-hour culture of the strain A-12 of *P. pestis* grown on Bacto blood agar base at 26°C was suspended in 0.06 M phosphate buffer (pH 7.2). The synthetic medium inoculated with 2.5 ml of this suspension which contained approximately 1 x 10^9 cells per ml, was incubated at 37°C on a reciprocating shaker for 24 to 96 hours. During growth the pH was adjusted as required to 7.0-7.2 by the addition of either 2 N HCl or 2 N NaOH. Five ml of 25% xylose added twice daily also served to control the reaction. Initially the pH of the culture rose to 8.2-8.5 but after the first adjustment it generally remained near neutrality for at least 96 hours. After incubation the cultures were centrifuged at about 15,000 G for 30 minutes in a Servall SS-2 centrifuge at approximately 10°C. The supernatant was filtered through a sintered glass candle of UF porosity and to 50 parts of filtrate was added one part of sterile 5.0% potassium aluminum sulfate. The mixture, adjusted to pH 6.0 to facilitate precipitation, was left at 4°C overnight and the precipitate collected by centrifugation was washed 3 times with sterile 0.85% saline solution and finally resuspended in a volume of saline (containing 1:5000 merthiolate) equal to 1/50 or 1/25 the original amount of filtrate. To test the immunogenicity of the alum adsorbed antigen, various amounts of the material were inoculated into each of a group of 10 mice; each animal of a group received only one dose. One week after immunization the mice were challenged intraperitoneally with approximately 500 cells (100 LD_{50}) of

TABLE II. Protective Effect of Filtrates Obtained from Cultures of *Pasteurella pestis* Strain A-12, Grown in a Synthetic Medium when Injected into Mice (Namru strain).

Culture No.	Culture Hr incubation	ml inj.*	Survival out of 10	ED_{50},† ml
1.1	48, aerated	10.3	9§	
1.2	48, static	9	0	
2.0	24, aerated	10	1	
		2	1	
		.4	0	
3.2	88, "	10	10	
		2	8	
		.4	9	
3.1	72, "	10	10	
3.3	48, "	10	6	5.0
		2	2	
		.4	3	
4.1	92, aerated, containing .01 M $MgSO_4$ and .01 M Na-citrate‡	10	8‖	
		2	9	
		.4	9	
4.3	88 (*idem*)	7	10	
		1.4	10	
		.28	8	
4.2	42 (*idem*)	10	10	.4
		2	9	
		.4	5	
5.1	94, .02 M $MgSO_4$	5	8§	
		1	8	
		.2	7	
5.2	94, .01 "	5	6§	
		1	7	
		.2	9	
5.3	94, .005 "	5	8	
		1	7	
		.2	8	
5.4	94, .0025 "	5	9	.2
		1	8	
		.2	5	

Except where noted, filtrates were treated with 5% alum.

* Alum or phosphate adsorbed antigen expressed as ml of whole filtrates.

† Determined by method of Litchfield and Wilcoxon(3).

‡ Filtrate treated with 10% $CaCl_2$ and 10% $Na_2HPO_4 \cdot 12H_2O$.

§ Survival out of 9 mice tested.

‖ " " " 8 " " .

a virulent strain of *P. pestis* suspended in 0.1 ml phosphate buffer. Untreated mice were infected simultaneously with the test animals and 90.0 to 100% of these control animals succumbed to the infection. The mice were observed for 2 weeks following challenge and a portion of those that died were autopsied and cultures prepared from the spleen.

Results. The results shown in Table II indicate that with the exception noted below, all of the preparations were able to confer protection. It appeared that in static cultures insufficient antigen was produced within 48 hours to be detected by mouse assay. Similarly, preparations from 24-hour shaken cultures did not protect mice with amounts used. By 42 hours, however, sufficient antigen was produced in shaken cultures so that in one case (filtrate 4.2) the ED_{50} as determined by the method of Litchfield and Wilcoxon(3), was 0.4 ml of filtrate. With another preparation (filtrate 3.3) obtained from a 48-hour shaken culture the ED_{50} was 5.0 ml while with a third culture filtrate, 4.1, the ED_{50} was 0.2 ml after 92 hours incubation. In the remaining tests the survival ratio (survivors/total number tested) was too high to furnish data appropriate for the computation of the ED_{50}.

The medium described in Table I was extremely turbid as a result of the formation of insoluble magnesium phosphate. By decreasing the concentration of $MgSO_4$ from 0.02 M to 0.005 M the turbidity was reduced considerably with no apparent effect on the production of protective antigen (filtrate 5.1, 5.2, and 5.3, Table II). The precipitation of phosphates could also be eliminated by the incorporation of 0.01 M sodium citrate in the medium, however, the citrate inhibited the precipitation of alum. An attempt was made to concentrate the protective substances by adsorption onto calcium phosphate formed by the addition of 10.0% $CaCl_2$ and 10.0% $Na_2HPO_4 \cdot 12\ H_2O$ to the filtrate. Preparations 4.1, 4.2, and 4.3 listed in Table II were treated in this manner and each, on assay, induced a degree of protection of the same order as the alum adsorbed filtrates.

Injection into mice of the supernatants obtained following adsorption of the protective substances indicated that the immunizing antigens were removed incompletely with alum or calcium phosphate. These supernatants were toxic for mice when injected subcutaneously and the majority of the animals died within one to 3 days. Adsorption on these salts may serve to separate toxic components from the immunogenic substances.

Serological studies of the filtrates obtained from the cultures of *P. pestis* showed the presence of Fraction I of Baker *et al.*(4). Whether Fraction I antigen is solely responsible for the protection afforded mice cannot be assumed at present since other antigens were also detected.

Summary. These studies show that the soluble substances produced by *P. pestis* when grown at 37°C in a completely defined medium are capable of producing a high degree of protection when injected into mice. Further studies to determine the optimal conditions for growth and antigen production are necessary and are in progress.

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Relation between Time of Conditioning of Host and Survival of Tumor Homografts in Mice.* (21025)

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Successful and progressive growth of homografts of a transplantable tumor has been induced in normally resistant mice of an inbred strain by pretreating the hosts with killed homologous tumor tissue, normal serum, or antiserums to the tissues(1-4). (The term *homograft,* as used here, denotes a graft between animals of the same species, but of differing genetic backgrounds.)

The present investigation, one of a series directed toward elucidating the processes underlying the abrogation of resistance, deals with a study of the necessary time relationships, between treatment (conditioning) of the otherwise resistant host and inoculation of the tumor homograft, for successful survival of the graft. It was motivated by the observation that tumor homografts often exhibit marked growth before regression.

Materials and methods. The transplantable tumor was Sarcoma I, which is indigenous to the A strain of mice. When inoculated subcutaneously, it grows progressively in 100% of these mice, killing the hosts within 3 to 5 weeks. Hosts were from the C57BL/6Ks and C57BR/aSn strains. They are genetically unrelated to the A strain, and also differ from each other so that transplantable tumors indigenous to one strain will not survive in the other. Sarcoma I rarely survives in untreated mice of these strains. All mice were approximately 2 to 4 months old at the start of the experiments. The hosts were conditioned by intraperitoneal injections of supernatant of a centrifuged homogenate of Sarcoma I prepared according to a method previously described(5). Whole tumor, stored in the frozen state (–23°C), was homogenized with an approximately equal weight of 0.85% NaCl in a high-speed blendor. The homogenate was then centrifuged at 4000 x g for 20 minutes at a temperature of 4°C. The supernatant was recentrifuged at room temperature for 20 minutes at an average RCF of 11,000 x g. The second supernatant was stored frozen (–23°C) until needed. The grafting of live tumor was done by trocar under aseptic conditions. A small bit of tumor was placed subcutaneously in the interscapular region. The course of growth of the grafts was followed by periodic palpation until the animals either died with a progressively growing tumor or showed no gross signs of a graft for a consecutive period of 2 months, at which time they were sacrificed as negative.

Experimental findings. The time of survival of homografts of Sarcoma I in the untreated host was first determined. This was done by excising the subcutaneous bed in the region of the tumor inoculum, and regrafting

TABLE I. Viability of Homografts of Strain A Tumor, Sarcoma I, in Untreated C57BL/6Ks and C57BR/aSn Mice as Determined by Regrafting into Strain A/Ks Mice.*

Elapsed time between homografting and excision for regrafting (days)	No. of viable homografts as shown by No. of A/Ks recipients dying with tumors received from†	
	C57BL/6Ks donors	C57BR/aSn donors
3	10/10	10/10
7	10/10	10/10
10	9/10	10/10
15	4/10	0/10
21	1/12	—

* Bits of live tumor inoculated subcutaneously by trocar into the foreign strains (C57BL/6Ks and C57BR/aSn Mice). Tumor bed excised at stated number of days after inoculation and regrafted into mice of the A strain to test for viability.

† Numerators represent total No. of mice dying with tumors; denominators, total No. of mice grafted. Each A/Ks recipient received a graft from one donor.

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this material subcutaneously into mice of the indigenous A strain. Regrafting was done under aseptic conditions by nicking the skin in the mid-dorsum with scissors to provide a point of entry, and placing the inoculum subcutaneously with a pair of forceps. One strain A/Ks host was used for each C57BL/6Ks or C57BR/aSn donor. Survival in the foreign host was determined by positive growth of the regrafted tumor in the strain A recipient. (This test for survival was suggested by a similar procedure described by Mitchison(6).)

It is clear that tumor cells will survive at least as long as 10 to 15 days, and probably longer in the untreated foreign hosts (Table I). Actually there must be a longer survival period than is here indicated since it has been determined(7) that there is a given minimum number of cells necessary in an inoculum to obtain growth even in mice of the indigenous strain.

The effect of varying the time of injection of tumor supernatant (with respect to the time of grafting of live tumor) on the survival of tumor homografts is shown in Table II. A single injection of one ml of tumor supernatant was given each mouse on the days indicated in the table. It is clear that the normal resistance of the host is abrogated only if the animals are conditioned prior to tumor grafting, or, for the C57BR/aSn mice, at most one day after grafting, even though the grafts can be assumed to survive for as long as 2 weeks or more (Table I).

TABLE II. Relationship between Time of Conditioning of the Host and the Survival of Tumor Homografts.

Interval between inj. of tumor supernatant and grafting of tumor*	No. of mice dying with progressively growing tumors† C57BL/6Ks hosts	C57BR/aSn hosts
Days before grafting		
7	10/11	6/10
5	6/11	3/ 9
3	5/11	5/10
1	6/11	15/20‡
0	—	2/10
Days after grafting		
1	0/11	3/19‡
4	0/11	0/ 9
7	0/11	0/10

* Tumor supernate, derived from homogenate of Sarcoma I, administered as a single intraperitoneal injection.

† Numerators are No. dying; denominators, total No. of mice in each group.

‡ Data from 2 exp.

TABLE III. Time of Duration of Conditioning as Measured by the Growth of Strain A Tumor, Sarcoma I, in C57BL/6Ks Mice.

Time interval between inj. of tumor supernatant and grafting of live tumor	No. of C57BL/6Ks mice dying with tumors Treated	Uninj. controls
10 days	19/20	1/10
6 wk	8/10	1/10
10	8/10	0/ 6
14	12/12	0/ 6
19	10/10	0/ 6
23	6/11	0/ 6
28	7/10	1/12
32	9/12	0/ 6
36	5/10	0/ 6
40	5/10	0/ 6

The duration of the conditioning effect (Table III) was determined in C57BL/6Ks mice by inoculating different groups of mice with live tumor at increasing time intervals after treatment with tumor supernatant. The mice were from a group of 300 animals that had been used to produce antiserum to Sarcoma I incidental to other experiments to be reported elsewhere. In April 1953, they had received 4 intraperitoneal injections per animal of 0.5 ml each of tumor supernatant given over a consecutive period of 2 weeks. A group of 20 of these mice received grafts of live Sarcoma I 10 days after the last injection of the tumor supernatant, and thereafter groups of 10 to 12 mice each were grafted at approximately monthly intervals. Tumor was grafted simultaneously into untreated control mice of comparable age. At the time of this writing, progressive growth of homografts is evident in 6 of 11 mice which had been inoculated with live tumor 45 weeks after being injected with the tumor supernatant.

Discussion. Our data indicate that the normal immune reactions to the tumor homografts are negated only if the animals are conditioned prior to tumor inoculation. Since the homografts survive at least for as long as 7 days in all the untreated mice, tumor super-

natant injected as recently as one day before tumor grafting would have a sufficient period to initiate the processes (which may require some time for their development) leading to abrogation of resistance in the host. In this sense, the conditioning process resembles the time relationship observed in the preparation of animals to produce immune sera. The extremely long period over which the effects of conditioning persist is of interest in this connection. It is hardly likely that tissue antigens would survive in the host over so long a time (at least 45 weeks in these experiments). If an immune reaction is involved in conditioning (and at present this is a moot point), the inoculation of the live tumor graft may elicit an anamnestic response.

Summary. In previous studies, it has been shown that in many instances the normal resistance of mice of an inbred strain to homografts of a tumor can be abrogated by injecting the host with an extract of tumor tissue. It is here shown that such an injection must be given prior to tumor inoculation, or at most one day after inoculation, to obtain loss of resistance. The effects of such a procedure persist for long periods, as shown by progressive growth of tumor homografts in 80-100% of mice grafted approximately 5 months after receiving injections of tumor extract, and in at least 50% of the mice grafted up to 10 months after treatment with extract.

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Vitamin B_{12} Activity of Plasma and Whole Blood from Various Animals.* (21026)

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Numerous reports have recently appeared concerning the vit. B_{12} activity of whole blood (1-4) and blood plasma or serum(5-8) in a variety of animal species. With the exception of the study by Yamamoto *et al.*(9) which indicated that dog erythrocytes contain no microbiological B_{12} activity, information concerning the distribution of vit. B_{12} between the erythrocytes and plasma of blood is lacking.

It seemed of interest, therefore, to study the distribution of vit. B_{12} activity between the cellular fraction and plasma of blood from a variety of animals including mammals, birds and reptiles. The results of this investigation are presented in this report.

Materials and methods. Blood was obtained from the human, rabbit, dog, calf, chicken and alligator in oxalated or heparinized containers. The samples were divided into two portions and one of the portions was

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TABLE I. Total Vit. B_{12} Activity of Whole Blood and Plasma from Various Animals.

Animal	Sample	No. of determinations	Vit. B_{12} activity (μg/ml) Before alkaline treatment	After alkaline treatment	Activity destroyed
Human	WB*	6	.38 ± .05†	.12 ± .01	.26
	P	5	.16 ± .03	0‡	.16
Dog	WB	7	.33 ± .04	.11 ± .01	.22
	P	11	.29 ± .04	.13 ± .01	.16
Calf	WB	9	.31 ± .03	.10 ± .01	.21
	P	9	.10 ± .01	0	.10
Rabbit	WB	6	43.3 ±5.8	2.68 ± .35	40.6
	P	6	31.3 ±7.9	.50 ± .17	30.8
Chicken	WB	6	8.59 ± .65	2.06 ± .24	6.53
	P	7	1.19 ± .40	.13 ± .05	1.06
Alligator	WB	3	7.14 ±1.51	5.48 ±1.46	1.66
	P	8	.06 ± .01	.04 ± .01	.02

* WB = Whole blood. P = Plasma.
† Stand. error.
‡ Values less than 0.01 μg/ml cannot be detected.

used to provide plasma. Hematocrits were determined in the usual manner using conventional Wintrobe hematocrit tubes. The plasma samples were assayed for vit. B_{12}§ activity before and after alkaline hydrolysis by the procedure of Rosenthal and Sarett(8) using *Lactobacillus leichmanii* 4797 as the test organism. Whole blood vit. B_{12} activity was determined with a slight modification of the above procedure. Although the greater bulk of whole blood proteins was removed by heating a 1:6 dilution of blood at pH 5.1, some acid soluble protein remained. This residual protein was found to precipitate readily when a suitable aliquot of the initial acid extract was adjusted to pH 6.8 ± 0.1, and diluted with distilled water to a final concentration of 1:10. The precipitate was then removed by centrifugation and the clear supernatant assayed for vit. B_{12}. In order to release desoxyribosides and vit. B_{12} from nucleoprotein(3), whole blood and plasma from the chicken and alligator was diluted 1:4 with distilled water, layered with toluene and autolyzed at 37°C for 36-44 hours. Since it was found in preliminary experiments that the vit. B_{12} activity of mammalian blood was not increased significantly by autolytic procedures, autolysis of the samples was omitted.

Results. The vit. B_{12} activity of whole blood and plasma from the various animal species before and after alkaline hydrolysis, is shown in Table I. With the exception of the rabbit, the vit. B_{12} activity of whole blood for the mammalian species is similar before and after alkaline hydrolysis. It is of special interest to note the high vitamin content of rabbit blood which contains more than 100 times the vitamin content of any other mammal studied. The whole-blood values for the human, calf and dog, are slightly lower than those previously reported by other workers (1), while the values for the rabbit are higher. The differences reported here may be due to dietary and genetic factors, as Anthony *et al.* (4) have shown in cattle. These workers found variations in blood vit. B_{12} activity not only between different strains of cattle but between groups of cattle of the same strain when fed different rations.

The mammalian plasma vit. B_{12} activity is lower than that of the corresponding whole blood, and again, rabbit plasma contains much more vit. B_{12} than does plasma from the other mammals. The values for the vit. B_{12} activity of human and rabbit plasma are in good agreement with the activity found by Ross

§ In this report, the term vit. B_{12} is restricted to denote material which is microbiologically active for *L. leichmanii* 4797 and is destroyed by alkaline treatment. The term vit. B_{12} activity denotes all substances which are microbiologically active for this organism including the desoxyribosides of guanine, thymine, hypoxanthine and others.

TABLE II. Vitamin B_{12} Activity of Blood and Plasma from the Chicken and Alligator.

Animal	Sample*	Vit. B_{12} activity (μg/ml) Before autolysis — Before alkaline treatment	Before autolysis — After alkaline treatment	After autolysis — Before alkaline treatment	After autolysis — After alkaline treatment
Chicken	WB	3.16 (6)†	4.18 (6)	8.59 (6)	2.06 (7)
	P	.98 (2)	.09 (6)	1.19 (7)	.13 (7)
Alligator	WB	2.77 (4)	2.95 (4)	7.14 (3)	5.48 (3)
	P	.04 (7)	.02 (7)	.06 (8)	.04 (8)

* WB = Whole blood. P = Plasma.
† The number in parentheses indicates number of determinations performed.

(5) who used *Euglena* as the test organism.

In mammalian whole blood, some of the vit. B_{12} activity is resistant to alkaline hydrolysis and is presumably due to desoxyribosides(3,10). The blood of rabbits contains a larger quantity of the alkaline stable material than does that of the other mammals. This amount, however, represents less than 6% of the total vit. B_{12} activity of rabbit blood and approximately 30% of the activity in the blood of the other mammals. In contrast to whole blood, plasma from the human and calf contains practically none of the alkaline stable material and only small amounts of these substances are present in the plasma of the dog and rabbit.

Whole blood from the chicken and alligator (after autolysis) contains more vit. B_{12} activity than any of the mammals with the exception of the rabbit. The alkaline stable material comprises 24 and 77% of the total vitamin activity of the chicken and alligator blood respectively. Plasma from these animals contains considerably less vit. B_{12} activity than does whole blood. The plasma vitamin activity of the chicken is greater than that of any of the mammals with the exception of the rabbit, while the plasma vitamin activity of the alligator is barely detectable. The residual plasma alkaline stable material in the chicken and alligator is similar to that found in the human, calf and dog.

The apparent vit. B_{12} activity of whole blood from either the alligator or the chicken was increased considerably when the blood was subjected to autolytic procedures (Table II). Similar analysis of the plasma from these animals shows that autolysis releases very little additional vit. B_{12} activity. The vit. B_{12} content of blood is, therefore, presumably associated with the nuclei of the cellular fraction of blood (primarily the erythrocyte fraction) and becomes available to the test organism only after the enzymatic action of autolysis.

It was not possible to determine the proportion of vit. B_{12} present in whole blood prior to autolysis since alkaline treatment to destroy the vitamin invariably released greater amounts of vit. B_{12} activity than was present in the original sample. The additional alkaline stable material is presumably due to liberation of desoxyribosides from the alkaline hydrolysis of nucleoproteins.

The distribution of vit. B_{12} between the plasma and the erythrocytes (Table III) was calculated from the hematocrit and the data for whole blood and plasma vit. B_{12}. For the mammals (with the exception of the calf) the vitamin is almost equally distributed between the erythrocytes and plasma within the limits of experimental error. In contrast to the mammalian species, the vit. B_{12} content of the blood from the chicken or alligator is largely present in the cellular fraction. This activity may be associated with the high nucleic acid content of the nucleated erythrocyte of these animals.

It is apparent from these data that vit. B_{12} is intimately associated with the cellular fraction of blood derived from animals containing nucleated red cells. In those animals in which a nucleated erythrocyte is not a normal constituent of the blood, the vitamin is almost equally distributed between the cells and the plasma. The slightly greater concentration of vit. B_{12} in the cellular fraction of blood from mammals may be due to the amount of

TABLE III. Distribution of Vitamin B_{12} in the Blood of Various Animals.

Animal	Hematocrit (%)	Whole blood vit. B_{12} (μg/ml)	Plasma vit. B_{12} (%)	Erythrocyte vit. B_{12} (%)
Human	38	.26	38.4	61.6
Dog	45	.22	40.9	59.1
Calf	40	.21	28.6	71.4
Rabbit	36	40.6	48.5	51.5
Chicken	29	6.53	11.6	88.4
Alligator	21	1.66	1.2	98.8

vitamin contained in the leukocytes and young forms of red cells. This is especially true of the calf since it is known that immature mammals may contain large numbers of immature, nucleated erythrocytes(10).

Summary. Vit. B_{12} activity of whole blood and plasma from the human, dog, calf, rabbit, chicken and alligator was studied before and after alkaline hydrolysis. The data show a wide variation of blood and plasma vit. B_{12} content between the different species. In mammals, the vitamin is almost equally distributed between the erythrocytes and plasma. In chicken and alligator, the major portion of the vitamin activity is associated with the nucleated erythrocyte.

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Pharmacological Studies with Rescinnamine, a New Alkaloid Isolated from *Rauwolfia serpentina.* (21027)

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The favorable clinical results obtained with preparations of *Rauwolfia serpentina* have created considerable interest in the active principles of this root and their mechanism of action. While at least 8 alkaloids had been found and investigated by various Indian and European workers, the only highly potent alkaloid found was reserpine, first isolated and studied by Müller, Schlittler, and Bein (1,2). In a recent communication from our Laboratories(3) it was stated: "Reserpine is the most potent single alkaloid so far examined. Available evidence suggests that other active alkaloid(s) are present in *Rauwolfia serpentina.*"

Since the known compounds account for only about 50% of the alkaloids in Rauwiloid®,* it was logical to look for additional active principles in the amorphous portion of this preparation. From it Klohs and coworkers(4) succeeded in isolating and identifying a new crystalline alkaloid, the trimethoxycinnamic ester of methylreserpate (M.P. 238-9°, $[\alpha]_D^{24}$ −97 ± 2, c, 1.0 in $CHCl_3$). As will be shown in this communication, rescinnamine has all the typical pharmacologic properties of Rauwiloid® and reserpine. The new al-

* The alseroxylon fraction of *Rauwolfia serpentina,* is a concentrate of the active alkaloids of the root, free from non-alkaloidal matter.

TABLE I. Observations in Dogs after a Single Intravenous or 5 Daily Oral Doses of Rescinnamine or Rauwiloid®.

Dose (γ/kg) and route	No. of dogs	HR*	MAP*	Sed.†	NM†	Dia.†
Controls	56	145 ± 37	117 ± 18	0/56	0/56	0/56
Rescinnamine						
250 i.v.	3	98 ± 19	69 ± 8	3/ 3	3/ 3	3/ 3
50 "	3	106 ± 25	81 ± 9	3/ 3	3/ 3	3/ 3
25 "	3	136 ± 25	93 ± 9	0/ 3	0/ 3	1/ 3
10 "	3	104 ± 12	99 ± 12	0/ 3	1/ 3	1/ 3
5 "	3	104 ± 18	106 ± 26	0/ 3	1/ 3	0/ 3
50 oral	10	76 ± 23	80 ± 9	9/10	10/10	10/10
15 "	10	83 ± 25	85 ± 8	4/10	10/10	9/10
5 "	9	91 ± 25	76 ± 15	0/ 9	4/ 9	5/ 9
1 "	10	112 ± 25	104 ± 20	0/10	6/10	3/10
Rauwiloid®						
1000 oral	15	93 ± 25	76 ± 15	13/15	13/15	13/15
250 "	13	85 ± 23	84 ± 16	5/13	12/13	12/13
50 "	10	111 ± 24	95 ± 13	0/10	3/10	1/10
5 "	10	120 ± 24	103 ± 12	0/10	7/10	7/10

* HR (heart rate in beats/min.) and MAP (mean arterial pressure in mm Hg) measured under pentobarbital and expressed as mean ± stand. dev.

† Animals showing sedation (Sed.), nictitating membrane prominence (NM), or diarrhea (Dia.)/animals tested. These data were recorded before anesthesia was induced.

kaloid when given orally or intravenously produces in normotensive dogs bradycardia, hypotension, and at higher doses also sedation. At higher doses, nictitating membrane prominence and diarrhea are also seen in animals. Rescinnamine also produces the same alterations in cardiovascular responses which have been demonstrated for Rauwiloid® by Gourzis *et al.*(5). In brief, these involve reduction or abolition of pressor responses to certain classical laboratory stimuli. The prolongation of pentobarbital-induced sleeping time of mice which has been suggested as an index of sedative activity of Rauwiloid®(6) is also observed with rescinnamine.

Methods. Dogs, unselected as to sex, weight or breed were medicated orally or intravenously. For oral use, the drug was given in capsules daily for 5 days. For parenteral administration the drug was dissolved in a few drops of glacial acetic acid and diluted to the required volume with distilled water. The pH of the final solution varied from 3 to 5. Twenty hours after the intravenous injection or on the sixth day of oral feeding, sedation was estimated using spontaneous activity and response to stimulation as criteria. Diarrhea and nictitating membrane prominence were noted. The dogs were then anesthetized with pentobarbital sodium intravenously, disappearance of the swallowing reflex serving as the end point. Twenty minutes after anesthesia had been established, a needle was inserted into the femoral artery and connected to a Sanborn electromanometer for recording of arterial pressure and heart rate. The recordings were made 10 minutes after the arterial needle had been inserted. In some of the orally medicated animals, responses to certain cardiovascular stimuli were determined using the procedure of Gourzis *et al.*(5). In another experiment dogs, anesthetized with urethane intravenously, were prepared for recording of MAP on the mercury manometer. Various reflex responses were measured before and after bilateral vagotomy. Rescinnamine was then administered intravenously and the responses were determined at hourly intervals up to 7 hours. For the sleeping time experiments, mice of both sexes (17-21 g) were used. Rescinnamine solution was injected intraperitoneally (0.01 ml/g of body weight). Two hours later pentobarbital sodium was given intraperitoneally at a dose of 65 mg/kg in 0.65% aqueous solution. The duration of sleeping time was judged by the righting reflex of the animals. All experiments were made in groups of 10 animals, including a

TABLE II. Cardiovascular Responses of Dogs under Pentobarbital Anesthesia following Oral Medication with Rescinnamine (50 γ/kg/Day for 5 Days).

	Medicated (9) dogs			Control (15) dogs†		
	Pre-ex. MAP, mm Hg	Max. MAP response mm Hg	Max. MAP response %	Pre-ex. MAP, mm Hg	Max. MAP response mm Hg	Max. MAP response %
Epinephrine, 1 γ/kg	104 ± 17	82 ± 23*	80 ± 24	127 ± 20	32 ± 13	25 ± 10
3 γ/kg	99 ± 17	126 ± 38*	127 ± 19	121 ± 15	70 ± 21	58 ± 17
Isuprel, 3 γ/kg	97 ± 22	−46 ± 21	−46 ± 14	133 ± 18	−54 ± 13	−41 ± 10
Carotid occlusion (bilateral, 30 sec.)	89 ± 26	10 ± 9*	11 ± 9	133 ± 18	36 ± 23	27 ± 17
Hypoxia (100 N_2, 45 sec.)	90 ± 21	−22 ± 13*	−25 ± 14	134 ± 16	+34 ± 23	25 ± 17

All figures given as mean ± stand. dev.
* Indicates significant difference ($p = 0.05$ or less) from the controls.
† Control values from Gourzis, *et al.*(5).

control group. The sleeping time of the medicated groups was expressed as percentage of that of the corresponding control group. This reduced uncontrolled daily fluctuations.

Results. The direct effects of rescinnamine after a single intravenous dose or repeated oral doses ranging from one to 250 γ/kg were bradycardia, hypotension, and, at higher doses, sedation (Table I). Nictitating membrane prominence and diarrhea were frequently observed. For comparison, results of 5-day oral Rauwiloid® tests are presented. The control data were accumulated over a 2-year period.

The new alkaloid was, on a weight basis, considerably more potent than Rauwiloid®. The changes in cardiovascular responses to epinephrine, Isuprel, carotid occlusion and hypoxia (Tables II and III) were also evoked by a smaller dose (50 vs. 500 γ/kg/day). Thus, rescinnamine is one of the highly active principles of Rauwiloid® and is of the same order of activity as reserpine(6).

The pressor response to carotid occlusion was significantly reduced on direct comparison. Applying the formula of Prochnik *et al.* (7), the orally medicated animals gave a response of 39 ± 24% vs. 51 ± 38% for the controls. For a group of 9 dogs, this difference is not significant at the 95% level.

The characteristic latency of Rauwiloid® action was shown by rescinnamine. After the drug was given intravenously to dogs anesthetized with urethane several hours had to elapse before significant changes occurred (Table III). About 3 to 4 hours after a single intravenous dose of 300 γ/kg the MAP had significantly dropped and the primary pressor response to central vagal stimulation disappeared. With doses of 100 γ/kg the delay was 4 to 6 hours. The hypoxia test was performed only once, 7 hours after injection of rescinnamine, when the pressor response to 100% nitrogen for 45 seconds was blocked in 2/4 and in 3/4 dogs following doses of 100 and 300 γ/kg, respectively. The response to bilateral carotid occlusion after 300 γ/kg was smaller at the end of 7 hours than it had been before the injection or in the controls. However, no change occurred according to the formula of Prochnik *et al.*(7). This type of experiment did not demonstrate bradycardia.

The prolongation of pentobarbital-induced sleeping time in mice (Table IV) was significant after 5 mg/kg of rescinnamine and it increased with larger doses. With the same doses, voluntary motor activity was greatly reduced within one hour after injection of rescinnamine.

The incidence of eyelid ptosis in mice has been suggested by Rubin and Burke(8) as the basis for a bioassay of reserpine-like substances in *Rauwolfia serpentina*. Rescinnamine produced ptosis in mice within 2 hours after intraperitoneal administration of a minimum dose of 2 mg/kg. Probably more than 30 mg/kg are required to reach a maximum effect as indicated by complete closing of the eyes.

When given intraperitoneally to rats at a dose of 10 mg/kg, rescinnamine produced within 15 minutes a marked sedation and a continuous, copious nasal discharge in 3 animals tested.

TABLE III. Cardiovascular Responses of Dogs under Urethane Anesthesia Measured at Hourly Intervals following Intravenous Injection of Rescinnamine.

Test	Dose (γ/kg)	No. of dogs	Before medication: Pre-vagot.	Before medication: Post-vagot.	After injection of rescinnamine: 1 hr	2 hr	3 hr	4 hr	5 hr	6 hr*	7 hr*
Mean arterial pressure, mm Hg	100	4	154 ± 23	156 ± 34	147 ± 30	151 ± 39	168 ± 16	142 ± 24	139 ± 27	128 ± 37	119 ± 36
	300	5	142 ± 10	145 ± 8	146 ± 16	143 ± 11	131 ± 18	117 ± 18	106 ± 35	101 ± 40	91 ± 35
	C	7	134 ± 16	142 ± 26	131 ± 11	138 ± 17	146 ± 17	150 ± 17	151 ± 16	151 ± 8	144 ± 10
Bilat. carot. occlusion resp., mm Hg	100	4	50 ± 27	57 ± 25	63 ± 37	69 ± 33	92 ± 11	69 ± 26	65 ± 26	58 ± 36	49 ± 30
	300	5	66 ± 38	72 ± 24	83 ± 24	82 ± 22	54 ± 37	39 ± 23	47 ± 33	37 ± 27	23 ± 17
	C	7	36 ± 14	51 ± 17	58 ± 30	61 ± 30	69 ± 34	71 ± 36	72 ± 28	62 ± 31*	55 ± 13
Central vagal stimulation†	100	4		4/4 32 ± 27	4/4 29 ± 27	4/4 20 ± 12	—	2/4 9 ± 12	1/4 13	1/4 7	0/4
	300	5		5/5 74 ± 28	5/5 55 ± 15	5/5 33 ± 19	3/5 26 ± 11	3/5 16 ± 20	1/5 36	1/5 34	1/5 24
	C	7		7/7 35 ± 16	7/7 44 ± 29	7/7 41 ± 19	7/7 42 ± 23	7/7 45 ± 21	7/7 35 ± 14	7/7 48 ± 11	7/7 45 ± 9

All responses given as mean ± stand. dev.

* Only 3 control animals followed for 6 and 7 hr.

† $\frac{\text{No. of dogs with pressor rise}}{\text{No. of dogs tested}}$ and mean pressor rise.

C = Control.

Discussion. The new alkaloid, rescinnamine, has the typical pharmacological properties of Rauwiloid® and reserpine. The only apparent discrepancy is that while the absolute pressure response to bilateral carotid occlusion was reduced by rescinnamine, the changes were not significant when related to the pre-existing MAP according to Prochnik *et al.*(7). This is in contrast to results with Rauwiloid®(5). The discrepancy may be explained by one or several alternatives: 1. The number of animals was too small. 2. The dose of rescinnamine was too small. 3. At the time when the pressor response started to decrease markedly, the pre-existing MAP in some animals was below 80 mmHg and, therefore, the formula was no longer applicable.

An accurate potency relation between Rauwiloid®, rescinnamine, and reserpine is difficult to establish. Several criteria may be considered: 1. Hypotension in dogs has a very flat dose response curve and wide standard deviations. 2. Alterations in cardiovascular responses present even greater problems of quantitative evaluation. 3. Sedation in dogs is very difficult to quantitate. 4. Prolongation of pentobarbital-induced sleeping time in mice. 5. Ptosis of eyelids in mice. The last two criteria have apparently a more satisfactory dose response curve, but their correlation to the therapeutic use of these drugs has yet to be established. However, the available data indicate that, in animals, rescinnamine is considerably more potent than Rauwiloid® and of the same order of activity as reserpine.

It is not known whether different potency relations exist between Rauwiloid®, rescinnamine and reserpine for various indices.

Summary. 1. The pharmacological properties of a new alkaloid, rescinnamine, recently isolated from Rauwiloid®, have been described. Rescinnamine produced typical pharmacological effects of *Rauwolfia serpentina*: bradycardia, hypotension, and sedation, as well as characteristic alterations in cardiovascular responses. 2. Rescinnamine caused augmentation of pressor response to epinephrine, reversal of pressor response to hypoxia, diminution of pressor response to bilateral

TABLE IV. Effect of Rescinnamine on Pentobarbital-Induced Sleeping Time of Mice.*

Dose, mg/kg	No. of groups (10 mice ea.)	Sleeping time, % of controls
2.5	2	126
5.0	4	144 ± 17†
10.0	4	167 ± 3†
20.0	2	213 †

* Rescinnamine given intraperitoneally 2 hr before standard dose of pentobarbital. See text.
† Significant increase ($p = .05$ or less).

carotid occlusion, and blockade or reversal of primary blood pressure rise elicited by faradization of afferent vagus. 3. The sedative effects of rescinnamine were demonstrated by gross observation in dogs, rats and mice and by prolongation of pentobarbital-induced sleeping time in mice. 4. Rescinnamine produced marked eyelid ptosis in mice and a copious nasal discharge in rats. 5. On a weight basis, rescinnamine appeared to be several times as potent as Rauwiloid® and similar to reserpine. 6. Rescinnamine is the second highly potent alkaloid derived from *Rauwolfia serpentina.*

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Cultivation of Human Tissues in Media Containing Bovine Allantoic and Amniotic Fluids. (21028)

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Allantoic and amniotic fluids have been used in tissue cultures by a number of workers. Moppett(1), Wendrowsky, and Zapolska(2), and Grossfeld(3) employed amniotic fluid from the embryonated egg in drop cultures of chick tissues. Fedorow(4) used the fluid surrounding the embryo of the cephalopod Rossia as the medium in which to culture nervous tissue from this organism. Thomas and his coworkers(5) described the use of deproteinated bovine amniotic fluid for cultivation of the virus of foot and mouth disease in suspended cell cultures of foetal calf skin; and Enders(6) used whole bovine amniotic fluid in roller tube cultures of various human tissues, propagating a number of viruses in these cultures. Moppett(7) also advocated the use of chick embryo allantoic fluid in cultures of chick tissues. Since the healing of wounds appeared to be stimulated by extracts of embryonic tissues from species possessing a well-developed allantois, Robinson(8) considered that solutions of allantoin might be of benefit in the treatment of wounds, and clinical trials with commercially-prepared allantoin confirmed his view. As a result of his observation, Shipp and Hetherington(9) studied the effect of allantoin on drop cultures of chick heart tissue, but found no significant stimulation of fibroblast growth. Chu(10) used allantoin in greater concentration than these workers, and concluded that it had a slightly retarding action on the growth of chick epithelial cells and chondroblasts in culture.

During the collection of bovine embryonic fluid in this laboratory, it was frequently noted that blind puncture of the gravid uterus by means of a hollow trocar might result in the withdrawal of 2 kinds of fluid, differing in

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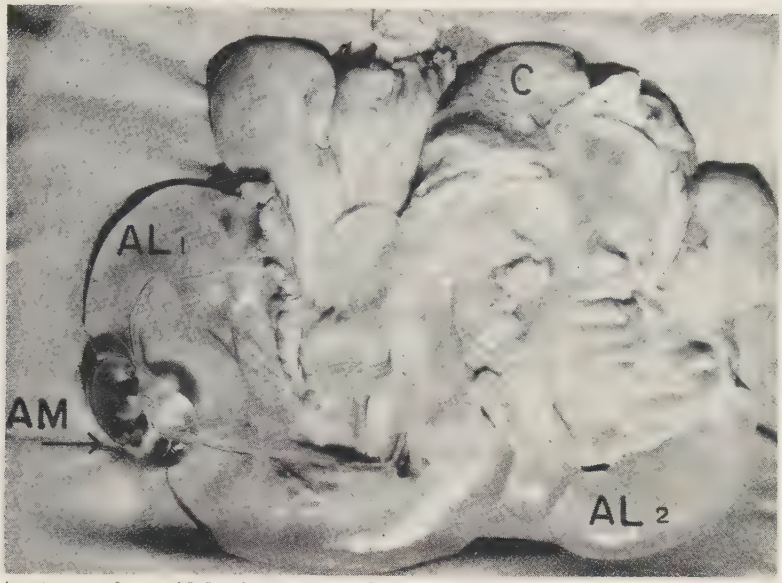

FIG. 1. Anatomy of gravid bovine uterus. C, cervix; AM, small amniotic sac—embryo 2 in. long; AL1, allantoic sac extension in pregnant horn; AL2, allantoic sac extension in non-pregnant horn.

appearance. Dissection confirmed that either allantoic or amniotic fluid, or both, could be obtained, depending on which sac had been penetrated. The present study was made to determine whether both fluids would support tissue growth; and the ability of Type 2 poliomyelitis virus to multiply in tissues cultured in either of these fluids was tested. Because of the usefulness of bovine amniotic fluid in the tissue culture field, a brief description of the anatomy of the gravid uterus is also presented.

Methods. Bovine uteri. These were used on the day of slaughter. A number were dissected to determine their anatomy; and fluids for the experiments were removed under direct vision from the allantoic and amniotic sacs. The embryos in the uteri examined measured up to 8 inches crown to rump. *Tissues.* Adult human uterus was used in Exp. 1. Skin and muscle from a 3-month human embryo were used in Exp. 2. *Tissue cultures.* Roller tube cultures were prepared by the method of Robbins, Weller, and Enders(11), and the medium was made up as follows:

Unfiltered allantoic or amniotic fluid	90%
Normal horse serum	5%
Bovine embryo extract	5%
Penicillin	100 units/ml
Streptomycin	100 μg/ml

0.1 ml of a stock 1% solution of soybean trypsin inhibitor was added for every 10 ml of medium. 2.0 ml of medium were put into each tube. The cultures were rotated at 37°C, and the medium was changed at intervals of 4 to 7 days, depending on the rate of metabolism of the tissues. *Growth measurement.* Tubes were examined daily under the microscope, using a 10× objective and a 5× ocular. The extent of outgrowth in each tube was estimated in millimeters, taking the average distance cells had spread radially from the edges of at least 6 explants. Very little difference was observed between the measurements for individual tubes in each group, and the results are therefore expressed as the mean for all tubes in each group.

Results. Anatomy of gravid uterus. The bovine uterus is bicornate, both horns participating in the development of the embryo (Fig. 1). *The chorion* lines both cavities, being attached to the uterus at a number of placental sites, or cotyledons; and umbilical vessels run along the lesser curvature of the embryonic sacs, with branches to the placentae. The chorion invests both the allantoic and the amniotic sacs, from which it can be separated by blunt dissection. *The allantoic sac* has 2 extensions connected by a narrower

TABLE I. Growth of Human Uterus Tissue in Media Containing Bovine Allantoic or Amniotic Fluid (Exp. 1).

Medium	No. of tubes	Mean outgrowth in mm on stated day after culture 3	5	8	10
Allantoic	5	0	.15	.35	.9
Amniotic	4	.1	.35	1.0	1.6

channel where it passes the amniotic sac. The longer extension may reach at least two-thirds of the way up the non-pregnant horn, and it is frequently possible to obtain a considerable volume of allantoic fluid from this horn, although it may occasionally be found that the contents of the sac in this situation are of a thinly gelatinous consistency and will not flow through a trocar. The uterine vessels accompany the sac into this horn. The second extension is in the tapering portion of the pregnant horn. Allantoic fluid may be clear in small embryos, but in larger specimens it tends to be opalescent or turbid, and on standing may form an amorphous deposit. Within the allantoic sac may be found detached bodies of a rubbery consistency, conforming to the description of hippomanes(12). As a rule, *amniotic fluid* is quite clear, and in older embryos it is darker than allantoic fluid, being of a yellow or yellow-green color. In the case of embryos under approximately 4 inches in crown-rump length, the amniotic sac is considerably smaller than the allantoic sac, and does not extend into the non-pregnant horn. There is thus a greater chance that allantoic fluid will be withdrawn from embryos of this size when blind puncture of the uterus is performed. In older embryos, distension of the amniotic sac obliterates any clear distinction between the broader parts of the 2 horns, and the allantoic sac is relatively much smaller.

Tissue growth. A. *Adult human uterus.* Table I summarizes the results of an experiment which demonstrated that while a medium containing allantoic fluid supported the growth of uterine tissue, the outgrowth was less extensive than that resulting from cultivation in amniotic fluid medium. In the tubes containing allantoic fluid the cells were somewhat granular, and outgrowth was less dense than in those containing amniotic fluid. It was also noted that in all the tubes with allantoic fluid the supporting plasma coagulum developed dark wrinkles or folds (Fig. 2) which tended to obscure the outgrowth. The outgrowths appearing in Exp. 1 on the 8th day after cultivation in the 2 kinds of fluid are shown in Fig. 2. The fluids used in this experiment were obtained from a single embryo measuring 7 inches crown to rump. Allantoic fluid was taken from the extension of the sac in the narrow portion of the pregnant horn, which contained about 200 ml of opalescent liquid. On standing, a white precipitate formed, and the material used consisted of the supernatant fluid after centrifugation at 1000 rpm for 5 minutes. The amniotic sac contained approximately one liter of clear yellow-green fluid from which after

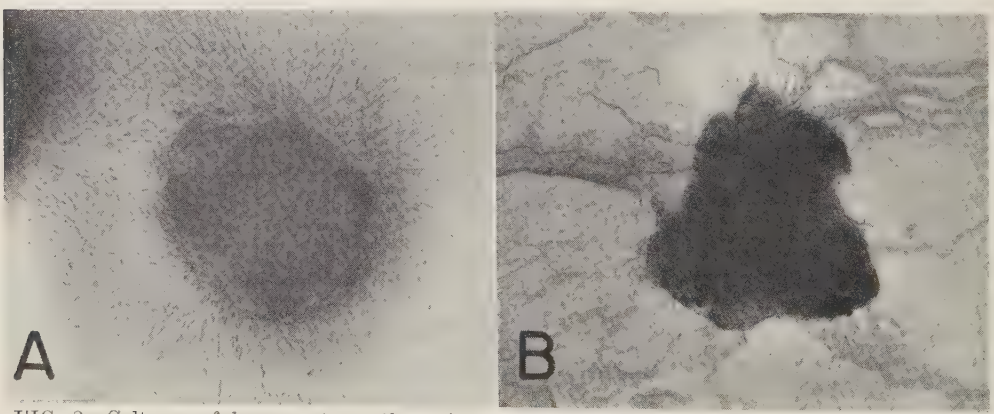

FIG. 2. Cultures of human uterus tissue in media containing bovine amniotic and allantoic fluids (Exp. 1). Outgrowths on 8th day after culturing. 25×.
A—amniotic fluid medium. B—allantoic fluid medium.

TABLE II. Growth of Human Embryonic Skin and Muscle in Media Containing Bovine Allantoic or Amniotic Fluid (Exp. 2).

Medium	No. of tubes	Mean outgrowth in mm on stated day after culture 2	3	4	5
Allantoic	7	.26	.53	1.0	1.4
Amniotic	7	.35	.7	1.4	2.1

withdrawal no solid material separated.

B. *Human embryonic skin-muscle.* In Table II are given the results of Exp. 2 in which tissue from a human embryo was grown in allantoic or amniotic fluid obtained from 2 bovine embryos measuring 6 inches crown to rump. The allantoic fluid was taken from the extension of the sac in the narrow end of the pregnant horn; it was opalescent but was not centrifuged prior to use. In the allantoic fluid tubes it was again noted that wrinkling of the plasma coagulum occurred, while the cells were granular and less numerous than in the amniotic fluid tubes. The amniotic fluid was transparent and yellow-green in color; and it supported the growth of clear and abundant cells while the coagulated plasma was not affected.

Cultivation of Type 2 poliomyelitis virus. Embryonic skin and muscle cultures from Exp. 2 were inoculated with 100 ID_{50} of Type 2 (Lansing) poliomyelitis virus. The media were replaced by allantoic and amniotic fluids without the addition of horse serum or embryo extract. Within 72 hours after inoculation approximately one-third of the cells in the inoculated tubes were destroyed. Further incubation resulted in progressive degeneration, while uninoculated control tubes remained unaffected.

Discussion. The above experiments indicate that, as a culture medium, bovine amniotic fluid is preferable to allantoic fluid. From a practical standpoint, however, it is not possible to be certain that fluid obtained by blind puncture of a gravid uterus does not contain some allantoic fluid, and it is probable that a small proportion will not significantly influence tissue growth. Relatively large amounts of allantoic fluid are associated with embryos up to a crown-rump length of about 4 inches, which, according to the data provided by Swett, Matthews, and Fohrman(13) would indicate an age of approximately 60-90 days.

Some information concerning the composition of bovine amniotic fluid is available(14). There is evidence(15) that in the later stages of gestation, fetal urinary excretion occurs into the amniotic sac; but the direct connection of the allantoic sac with the urachus suggests that excretory products will be found at an earlier period in allantoic fluid. These products may account for the difference in growth observed in the above experiments with human tissues cultured in allantoic and amniotic fluids.

Summary. 1. Growth of adult human uterus and human embryonic skin and muscle tissues in roller tube cultures is supported by media containing 90% of either bovine allantoic or bovine amniotic fluids. 2. Amniotic fluid medium produces a profuse outgrowth of clear cells, and does not affect the plasma clot; whereas allantoic fluid medium tends to produce wrinkling and darkening of the clot, and induces granularity in a less profuse outgrowth of cells. 3. Either fluid, used alone as the medium, permits the cultivation of Type 2 poliomyelitis virus.

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Use of Tissue Culture Mediums Sterilized with Gamma Radiation from Cobalt-60.* (21029)

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Recent interest in tissue cell cultivation, resulting primarily from its successful application to problems in virology, has stimulated considerable effort towards simplification and improvement of methods and materials. In spite of the increased use, however, the greatest difficulty confronting the tissue culture worker continues to be the lack of a simple medium which can be easily prepared and handled. A synthetic medium, capable of sustaining cell proliferation, has not yet been developed and almost all tissue culture mediums contain serum and embryonic extract as major components. While these materials may be sterilized by filtration the procedure is both tedious and uncertain.

The present paper is a preliminary report of studies designed to test the feasibility of using tissue culture mediums sterilized with gamma radiation from Cobalt-60. Though ample evidence is available that such radiations will sterilize a variety of biological materials under appropriate conditions(1-3) it was necessary to establish the fact that the particular materials in question here, namely serum and embryonic extract, could be sterilized in the form in which they are commonly used for tissue culture work. A second and more critical point was to determine the suitability of such irradiated mediums for cell cultivation and was concerned with: a) possible destruction of growth promoting properties, b) production of toxic substances or materials capable of stimulating abnormal growth, c) alteration of other significant physical or chemical properties. Since plasma is frequently used in tissue culture work to provide an imbedding matrix, it was substituted for serum in these initial studies. In this way it was possible to determine the effect of irradiation on the clot forming ability as well as on growth promoting properties. The effect of gamma radiation on the ability of embryonic extract to induce clot formation was also studied.

Materials and methods. Irradiation for this study was carried out with the large Cobalt-60 source of the Michigan Memorial Phoenix Project(4). This source develops 2.2 x 10^5 rep/hr in the center well: samples were irradiated in the center well and received total dosages varying from 1 x 10^5 to 1.2 x 10^6 rep. In all experiments, control samples were kept at approximately 15°C, the temperature of the irradiation chamber, for the duration of the exposure. Tests were made with *chicken plasma* and *embryonic extract*: they were used in the form of commercially dehydrated products (Difco) and as freshly prepared fluid samples. The former were irradiated both in the dehydrated state and following

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reconstitution with distilled water. All samples were irradiated in 5 ml quantities that were tightly sealed in 10 ml glass bottles. Following irradiation the samples were refrigerated at 4°C until tests could be performed. Aqueous embryonic extract was stored at −20°C. Twenty four-hour broth cultures of *E. coli* I, *Staph. aureus* RK2, and a recently isolated coagulase positive *Staph. aureus* were used to determine the ability of gamma radiation to sterilize tissue culture fluids. Samples were contaminated with known numbers of viable organisms and then irradiated in either the aqueous state or following dehydration.‡ In fluid specimens the numbers of viable organisms per ml were determined by plating 0.1 ml of each 5 ml sample in brain heart infusion agar both before and after irradiation. Plate counts were made after 48 hours and again after 72 hours incubation at 35°C. For dehydrated samples, the numbers of organisms per ml prior to irradiation were determined from control samples of the same lot held at room temperature. *pH* was determined on fluid samples before and after irradiation. The pH of dehydrated samples exposed to gamma radiation was measured following reconstitution and was checked against that of reconstituted control samples. All pH measurements were made with a Beckman model G pH meter using a microadapter cup(5). The *clotting properties* of chicken plasma samples were tested by adding 2 drops of "standard" embryonic extract to 4 drops of plasma in a 10 x 75 mm tube. Both coagulation time and consistency of the clot were evaluated. In the case of dehydrated plasma, a non-irradiated sample from the same production lot served as a control. The ability of embryonic extract to induce clot formation of a "standard" plasma sample following irradiation was tested in the same way. For the study of growth promoting properties explants from the ventricle of 9- to 11-day chick embryos were embedded in a plasma clot on 11x22 mm coverglasses. The latter were inserted in Agarslant culture tubes§ and overlaid with a medium containing 40% horse serum or human ascitic fluid, 50% Hank's balanced salt solution, and 10% chick embryonic extract. These tubes were incubated in a horizontal position at 35°C. Increase in area of outgrowth was measured using a calibrated ocular micrometer. At the end of the observation period the coverglasses were removed, the outgrowth fixed with Bouin solution, and then stained with Harris haematoxylin. The mitotic activity of the cells in the outgrowth was determined by microscopic examination of these stained slides.

‡ Preparation of dehydrated samples contaminated with known numbers of test organisms was kindly carried out by Dr. C. W. Christensen, Difco Laboratories.

TABLE I. Viable Bacterial Count as a Function of Radiation Dosage.

Dosage (rep) × 10^5	Coagulase positive *Staph. aureus* In balanced salt sol.	Coagulase positive *Staph. aureus* In aqueous EE*	*Staph. aureus* RK2 In dehydrated plasma	*E. coli* I In dehydrated plasma
0	1.1×10^6	4×10^6	390	230
1.0	21	125	0	0
1.5	—	11	—	—
2.0	—	0	0	0
2.5	0	0	—	—
3.0	—	0	0	0

* Embryonic extr.

Results. Table I shows that *E. coli* I and *Staph. aureus* RK2 in dehydrated chicken plasma were killed by 1 x 10^5 rep of gamma radiation when the starting inoculum was 2 x 10^2-4 x 10^2 organisms per ml. Aqueous embryonic extract contaminated with 4 x 10^6 *Staph. aureus* per ml was sterilized at 2 x 10^5 rep. In other experiments these organisms were destroyed by equivalent dosages when suspended in liquid plasma. It will be noted that the killing appears to be essentially the same for the organisms in embryonic extract or plasma as for a saline suspension of the

TABLE II. Clotting Time of Dehydrated Chicken Plasma following Irradiation.

Dosage (rep)	Duration, hr	Clotting time in min. Dehydrated	Clotting time in min. Dehydrated and reconstituted
0	Control	.8	1.0
2.2 × 10^5	1	.8	1.3
4.4 "	2	—	2.0
6.6 "	3	—	4.3
8.8 "	4	.8	5.0
1.8 × 10^6	8	1.3	—
2.2 "	10	1.3	—

§ Fisher Scientific Supply Co. (modified form).

TABLE III. Clot Induction Time of Aqueous and Dehydrated Chick Embryonic Extract after Irradiation.

Dosage (rep)	Duration, hr	Clotting time in min. Aqueous	Dehydrated
0	Control	1.5	.25–.33
2.2×10^5	1	1.5	.25–.33
4.4 "	2	1.5	—
6.6 "	3	—	.25–.33
8.8 "	4	1.5	.25–.33
1.3×10^6	6	1.5	—
1.8 "	8	1.5	—

organism.

Dehydrated plasma from one commercial lot was used in studying the effect of radiation on clotting properties and samples were irradiated both in the dehydrated state and following reconstitution with distilled water. The data in Table II show that as much as 8.8×10^5 rep was tolerated by plasma in the dehydrated state with no alteration of clotting time or consistency of the clot while samples irradiated in the liquid state showed a significant increase in clotting time following 4.4×10^5 rep exposure. Clotting time for one sample of fresh chicken plasma was increased from .5 to 1.3 minutes following exposure to 8.8×10^5 rep.

Irradiated samples were stored at 4°C for several weeks to check the possibility of changes occurring some time after irradiation, due to delayed chemical reactions. After 3 to 4 weeks storage the clotting time of these samples was determined and compared with that observed before and immediately following exposure to gamma radiation. In no instance was a significant alteration of clotting time observed under these conditions.

Both dehydrated and aqueous chick embryonic extracts were irradiated and subsequently tested for their ability to induce clotting of chicken plasma as shown in Table III. Repeated tests failed to show a change in clot induction time following exposure to doses of gamma radiation as high as 8.8×10^5 rep. One series of experiments failed to show any change in aqueous embryonic extract following irradiation of 1.8×10^6 rep. Measurement of pH of samples following irradiation in the dehydrated state showed a maximum change over the controls of 0.3 pH unit. The maximum change in fluid samples as determined by measurement before and after irradiation was 0.2 pH unit.

The ability of irradiated medium components to support normal growth of tissue cells was not impaired as shown by the data in Tables IV, V, VI. It will be noted in Table IV that embedding explants in irradiated plasma does not adversely influence growth of chick heart tissue as determined either by increase in area of outgrowth or by mitotic configuration. In fact, from these data, there would appear to be a stimulation of growth. This finding has not been a consistent one, however, and continuous growth for 6 weeks in clots formed from plasma exposed to 1.8×10^6 rep failed to alter the rate of growth or the mitotic configurations of chick heart explants (Table V). These cultures were carried through 4 transplantations.

As seen in Table VI, exposure of either aqueous or dehydrated embryonic extract to a gamma radiation dosage of 8.8×10^5 rep failed to interfere with growth or significantly alter the mitotic pattern of chick heart explants when the extract was used to induce clot formation and was added to the liquid phase of the medium in a concentration of 20%.

Discussion. The results of the preliminary

TABLE IV. Growth of 11-Day Chick Embryonic Heart in Irradiated Plasma.

Dosage (rep)	% increased area in 48 hr	Mitotic configuration (%) Resting	Separated nucleoli	Splitting nucleus	Prophase	Metaphase	Anaphase	Telaphase
0	100	39	52	7	2	0	0	0
2.2×10^5	423	47	45	6	1	1	0	0
4.4 "	412	44	43	8	4	0	1	0
8.8 "	830	48	47	2	1	2	0	1
1.3×10^6	1140	50	45	3	2	0	0	0
1.8 "	528	50	41	4	4	1	0	0
2.0 "	685	50	46	1	1	0	1	1

TABLE V. Continuous Growth of 11-Day Chick Embryonic Heart in Plasma Exposed to 2×10^6 Rep of Gamma Radiation.

Dosage (rep)	Mitotic configuration (%)						
	Resting	Separated nucleoli	Splitting nucleus	Prophase	Metaphase	Anaphase	Telaphase
0	67	23	9	0	0	0	0
2×10^6	74	24	0	0	0	0	2

TABLE VI. Growth of 9-Day Chick Embryonic Heart in Medium Containing Irradiated Embryonic Extract.

Sample	Dosage (rep) $\times 10^5$	% increased area, 48 hr	Mitotic configuration (%)						
			Resting	Separated nucleoli	Splitting nucleus	Prophase	Metaphase	Anaphase	Telaphase
Control	0	228	53	35	10	0	2	0	0
Aqueous	4.4	280	45	42	12	0	1	0	0
"	8.8	324	55	32	9	2	2	0	0
Dehydrated	8.8	139	54	38	7	1	0	0	0
"	8.8	249	29	60	8	1	2	0	0

studies presented here indicate that either fluid or dehydrated plasma and embryonic extract contaminated with non-sporeforming bacteria may be sterilized by gamma radiation from Cobalt-60 in dosages of 2 x 10^5 rep or less. Furthermore, irradiation of either dehydrated or liquid samples at this level does not alter the clot forming properties of plasma, the clot inducing capacity of embryonic extract, or the growth promoting properties of serum, plasma, or embryonic extract for embryonic tissues.

While the clotting time of plasma was significantly increased by exposure to 4.4 x 10^5 rep when in the fluid state, samples treated in the dehydrated form showed no change at 8.8 x 10^5 rep. This apparent protection was not due to complete absence of water as the commercially dehydrated samples used contained approximately 10% residual moisture. Dehydrated plasma exposed to 2 x 10^6 rep was still suitable for explantation of chick tissues and did not alter the growth pattern of chick embryonic heart tissue when cultures were carried in it for a period of 6 weeks.

As shown by Proctor and Goldblith(1), Lawrence, Brownell, and Graikoski(2), and others, the dosage of gamma radiation required to kill bacterial spores is considerably higher than that needed for vegetative cells. For several species of spore forming bacteria this is in the neighborhood of 2 x 10^6 rep when the organisms are suspended in physiological salt solution. Studies are in progress at the present time to determine the dosage required to destroy spores in serum, plasma and embryonic extract. However, it will be noted from the data presented in Tables IV and V that irradiation of plasma at 2 x 10^6 rep does not appear to make it unsuitable for cell cultivation. Recent studies, to be published later, indicate that such dosages may, indeed, be well tolerated.

Summary. Plasma, serum, and embryonic extract contaminated with gram negative and non-sporeforming gram positive bacteria have been shown to be sterilized by a dosage of 2 x 10^5 rep of gamma radiation from Cobalt-60. Such treatment failed to alter the properties of these materials as related to their use in tissue culture work. These studies are being extended to cover dosages necessary to kill bacterial spores and efforts are being made to put the work on a more quantitative basis by utilizing established strains of tissue culture cells for assay procedures.

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Effect of Streptomycin on Susceptibility of Intestinal Tract to Experimental Salmonella Infection.* (21030)

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Among the complications of antibiotic therapy in man are the "secondary infections" of the mucous surfaces with microorganisms insensitive to the drug being administered (1-3). Such infections are really a replacement of the normal microflora by new microbic populations which may or may not be disease producing, depending upon their virulence, *e.g.*, monilia(4,5) and gram negative bacilli(6) in the oropharynx, or staphylococci (7,8) in the bowel. These regions are constantly exposed to contamination with such microorganisms which, nevertheless, are rarely able to establish themselves, except during antibiotic therapy. The antibiotic is presumed to render such a site vulnerable to the implantation of contaminating microorganisms by suppressing or eliminating some of its normal inhabitants; *i.e.*, by disturbing the ecology of the microflora(9). The question therefore arises, to what degree does the normal flora of the oropharynx or bowel hinder the establishment of contaminating microorganisms and thereby assist the host in its defense against infection?

As the initial step in an investigation of this problem, experiments were undertaken to determine the effect of streptomycin on the susceptibility of the mouse's intestinal tract to infection with one of its natural pathogens, *Salmonella enteritidis*. Susceptibility was measured by determining the number of Salmonella required to infect mice by oral inoculation, the natural route for this microorganism. It was found that infection could be initiated by much smaller numbers of Salmonella after treatment with a large dose of streptomycin; *i.e.*, susceptibility to infection was markedly enhanced by preliminary treatment with this antibiotic.

Materials and methods. Inoculations were made by introducing known numbers of Salmonella in .5 ml broth directly into the stomach by means of a small bent tube attached to a .5 ml tuberculin syringe. The tube was made from an 18 gauge needle, 2″ long, the point of which had been removed and replaced with a bead of silver. The tip of the tube was passed carefully down the esophagus into the stomach. After sufficient practice it was possible to carry out this procedure with a minimum of trauma. Whenever there was doubt about the position of the tube, the mouse was discarded. After inoculation the mice were kept in isolation in individual jars to prevent cross-infection. *Administration of streptomycin*† was always by mouth. Single doses (in .5 ml saline) were injected directly into the stomach by the method just described. Similar injections of normal saline the day before inoculation in a group of controls ruled out the possibility that trauma was responsible for the increased susceptibility described below. When streptomycin was administered for several days, a fresh solution, changed daily, was added to the animals' drinking water. The mice were found to drink the streptomycin solution as readily as tap water. *The strain of S. enteritidis*‡ used was highly resistant to streptomycin. It was chosen because this property a) precluded its being mistaken for any other strains of Salmonella, b) facilitated its recovery and identification, and c) prevented its being affected by any streptomycin which might still be present in the bowel. The strain was originally recovered from the heart's blood of a mouse. It grew readily on agar containing 10 mg streptomycin per ml, but was not streptomy-

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† The streptomycin used in these experiments was kindly supplied by Abbott Laboratories; Merck & Co., Inc.; Chas. Pfizer & Co., Inc.; E. R. Squibb & Sons; and The Upjohn Co.

‡ For the type diagnosis of this and other strains of Salmonella the authors are indebted to the Division of Laboratories, Illinois Department of Public Health, Dr. H. J. Shaughnessy, Director.

cin-dependent. It was not highly virulent for this strain of mice, in which the LD_{50} (21 days) by intraperitoneal inoculation was 10^6. Virulence was maintained at this level by frequent passage through mice. *The mice* were Rockland "RAP" females,§ 9-10 weeks old, weighing 20-25 g. Their feed was Rockland mouse pellets. These mice were known to have been exposed to *S. enteritidis* because carrier surveys on representative numbers made at the time shipments were received in the laboratory showed a high incidence of positive fecal cultures, on occasion as high as 50%. It was found, however, that the feces of most of these carriers became Salmonella-free within a few days if the mice were segregated in individual jars. Illness and death attributable to Salmonella infection seldom occurred among stock mice, indicating a high degree of resistance—native or acquired. Although *S. typhimurium* was occasionally recovered from the feces of mice on arrival from the breeder, no epizootic caused by this microorganism occurred among the stock animals, although they were often held in reserve for several weeks, housed in groups of 32 to a cage. *Routine fecal cultures.* The mouse was put into a sterile 250 ml bottle, or a clean, unused (quart) cardboard container.‖ As soon as the mouse had defecated, the pellet of feces (average—20 mg dry weight) was transferred by means of a sterile platinum wire to a tube of 10 ml brain heart infusion broth containing 1 mg streptomycin per ml. The pellet of feces was mashed and evenly suspended by means of a sterile glass rod, flared at the end to fit the bottom of a test tube. Serial 10-fold dilutions were made in streptomycin broth. After 18 hours incubation, a loopful of each culture was streaked onto eosin-methylene-blue agar and/or brilliant green agar containing 1 mg streptomycin per ml. Autopsy cultures were made on 385 mice, including all of those tabulated in Table I. Before the mouse was killed, a pellet of feces was obtained and cultured by the methods just described. The mouse was chloroformed, autopsied under aseptic precautions and the heart's blood cultured in 10 ml brain heart infusion broth. The spleen was macerated in a tissue grinder, and cultured in broth. Positive cultures were streaked onto EMB and brilliant green-streptomycin agar. The entire gut was removed and ground in a Waring blendor with 50 ml streptomycin broth which was subcultured the following day onto EMB and brilliant green streptomycin agar.

As a check on the dependability of the method for detecting small numbers of Salmonella in the intestinal homogenate, the intestines of 20 normal mice were homogenized in streptomycin broth, pooled and divided into 50 ml aliquots. These were inoculated, in quintuplicate, with very small numbers of Salmonella, checked by plate counts and broth titration. The results showed that 1 or 2 microorganisms sufficed to initiate growth in the intestinal homogenate. Among the 264 experimental mice from which intestinal homogenate was cultured, the results in all but 8 confirmed those on a pellet of feces obtained shortly before the animal was killed. In these 8 instances (3%) the fecal cultures were negative and the intestinal homogenates positive.

Results. The results of a representative experiment are presented in Table I. Groups of 5 mice each were inoculated by mouth with the numbers of *S. enteritidis* indicated. The treated mice had been given 50 mg streptomycin by mouth 24 hours before inoculation. Not included in the table were uninoculated controls given 500 mg streptomycin without apparent ill effect. It will be seen that among the untreated mice no infection resulted from inoculations with fewer than 10^4 Salmonellae, and that among the 10 mice given the two largest inocula (10^4 and 10^5) only 4 showed Salmonella in their fecal cultures beyond the first day after inoculation. From only 3 of these 4 were Salmonellae recovered at the time of autopsy from intestinal homogenate and from the heart's blood and spleen.

Comparison of the foregoing with the results in the treated mice shows the effect of a single dose of streptomycin given 24 hours before inoculation. All treated mice inoculated with 42 or 420 Salmonellae became in-

§ Obtained directly from Rockland Farms, New City, N. Y.

‖ Made by Container Corporation of America.

TABLE I. Effect of a Single Dose of Streptomycin by Mouth on Establishment of Infection with *Salmonella enteritidis* 24 Hours Later.

	Fecal content of Salmonella* (logs)																	
	Streptomycin treated mice†									Control mice								
	Days after inoculation						Autopsy cultures			Days after inoculation						Autopsy cultures		
Inocula	1	2	4	6	8	11	Entire gut	Sp	HB	1	2	4	6	8	11	Entire gut	Sp	HB
.42	—	—	—	—	—	—	—	—	—									
	—	—	—	—	—	—	—	—	—									
	—	—	—	—	—	—	—	—	—									
	—	—	—	—	—	—	—	—	—									
	4	8	5	7	7	6		+	+									
4.2	—	—	—	—	—	—	—	—	—									
	4	—	—	2	—	—	—	—	—									
	4	2	4	5	6	—	—	—	—									
	5	4	7	6	3	6		+	+									
	2	7	5	4	5	7		+	+									
42.	5	8	5	4	4	5		+	—	—	—	—	—	—		—	—	—
	5	8	8	7	4	6		+	—	—	—	—	—	—		—	—	—
	3	4	6	6	6	7		+	—	—	—	—	—	—		—	—	—
	2	6	5	2	3	2		+	+	—	—	—	—	—		—	—	—
	6	5	7	6	5	7		+	+	—	2	—	—	—		—	—	—
420.	6	3	6	6	6	5		+	—	—	—	—	—	—		—	—	—
	3	6	8	5	4	4		+	+	—	—	—	—	—		—	—	—
	4	5	6	3	3	4		+	+	—	—	—	—	—		—	—	—
	2	5	4	5	3	3		+	+	—	—	—	—	—		—	—	—
	5	4	8	3	4	5		+	+	—	—	—	—	—		—	—	—
4.2×10^3										—	—	—	—	—		—	—	—
										—	—	—	—	—		—	—	—
										—	—	—	—	—		—	—	—
										—	—	—	—	—		—	—	—
										—	—	—	—	—		—	—	—
$\times 10^4$										—	—	—	—	—		—	—	—
										2	—	—	—	—		—	—	—
										2	—	—	—	—		—	—	—
										2	3	—	2	2	3	+	+	+
										2	4	4	4	3	3	+	+	+
$\times 10^5$										2	—	—	—	—	—	—	—	—
										4	—	—	—	—	—	—	—	—
										3	—	—	—	—	—	—	—	—
										3	3	2	2	3	—	—	—	—
										3	3	2	4	4	2	+	+	+

* Per pellet of feces. Numbers represent the log of the reciprocal of the highest dilution showing growth. —, culture negative for Salmonella. 2 indicates growth in the first tube only.
† 50 mg streptomycin introduced by mouth 24 hr before inoculation.

fected, as shown by the results of the routine fecal cultures and the positive cultures of heart's blood and/or spleen at autopsy. 6 of the 10 mice in these 2 groups had demonstrable Salmonella bacteremia. Definite infection also occurred in 2 of the 5 mice inoculated with approximately 4 Salmonellae, and in 1 of those with a 10-fold dilution of that suspension, indicated in the table as .42 Salmonella. The data also show that the numbers of Salmonella recovered from the serial fecal cultures (tabulated as the logarithm of the reciprocal of the highest dilution showing growth) were much greater (about 1000-fold) among the streptomycin treated mice than among the untreated controls.

Table II summarizes the results of all experiments in which mice had been treated with 50 mg streptomycin 24 hours before inoculation, and for comparison, the results on all untreated control mice. In those mice which were not autopsied for culture of blood, spleen and intestinal homogenate, the diagnosis of infection was based on the persistence of Salmonella in the serial fecal cultures beyond the 5th day after inoculation. It will be seen

TABLE II. Combined Results of Inoculations of Streptomycin-Treated and Control Mice.

No. Salmonella inoc.	Streptomycin-treated*			Controls		
	No. exps.	Total mice	Infected, %	No. exps.	Total mice	Infected, %
<1	4	36	14			
1 – 10	4	36	56	3	30	0
10 –100	4	36	83	7	66	1.5
10^2– 10^3	1	5	100	14	120	15
10^3– 10^4				8	97	27
10^4– 10^5				10	76	33
10^5– 10^6				5	47	50
10^6– 10^7				2	14	100

* 50 mg streptomycin by mouth 24 hr before inoculation.

that less than 10 Salmonellae sufficed to infect approximately half (56%) of the streptomycin treated mice and that 10^5 were required to infect half of the untreated controls. The ID_{50} (infective dose for 50%) was computed by Berkson's method(10)¶ to be 2.21 for the treated mice and 1.3 x 10^5 for the controls (see Table III). If it had been possible to autopsy all of the mice for evidence of visceral involvement and these results used as the criterion of infection, the ID_{50} would have been only slightly increased, since positive spleen cultures were obtained in 90% of the mice in which Salmonella persisted in the feces to the day of autopsy.

Effect of decreasing amounts of streptomycin. When the dose of streptomycin was reduced to 10, 7 or 5 mg, the effect produced was less pronounced, as measured by the ID_{50} of Salmonella. A dose of 1 mg was without effect.

Duration of the increased susceptibility to Salmonella infection following a single treatment of streptomycin. Groups of mice were treated by mouth with 50 mg streptomycin and at different times thereafter inoculated by mouth with graded doses of Salmonella. The numbers of Salmonella required to infect 50% (ID_{50}) were estimated by the method of Berkson. Despite the error inherent in the methods employed, the results presented in Table III show that susceptibility decreased with time after streptomycin treatment, but as long as 5 days after treatment mice still showed somewhat greater susceptibility than controls.

Effect of 50 mg streptomycin supplemented by continued treatment thereafter. Mice were treated with the standard dose of 50 mg streptomycin and thereafter given streptomycin in their drinking water in a concentration of 1 mg per ml for 3 or 4 days. The only effect of such additional treatment was an increase in the numbers of Salmonella recovered in the serial fecal cultures.

Discussion. These experiments were designed to make a rough quantitative estimate of the increase in susceptibility to an enteric infection which follows the administration of a large dose of streptomycin by mouth. This was done by determining the numbers of Salmonella required to initiate a definite infection in mice so treated and in untreated controls. The results show that the susceptibility of mice to infection with *S. enteritidis* inoculated by mouth, the natural portal of entry, was markedly enhanced by the oral administration of 50 mg streptomycin 24 hours before inoculation. Whereas approximately 10^5 Salmonellae were required by this method to establish infection in half of the untreated control mice, less than 3 microorganisms sufficed to infect 50% of the streptomycin treated mice. Moreover, the numbers of Salmonella recovered in the routine fecal cultures were much greater (about 1000-fold) in the mice which had been pre-treated with a single dose of streptomycin and still higher in the mice which had been given additional streptomycin for 3

TABLE III. Duration of Susceptibility following Oral Administration of 50 mg Streptomycin.

Days after treatment	No. mice	Infective dose 50*	95% confidence limit
1	113	2.21	1.18–4.13
2	48	3.97×10^2	1.03×10^2– 15.3×10^2
3	70	1.79×10^3	$.33 \times 10^3$– 9.61×10^3
4	60	2.3×10^4	$.037 \times 10^4$–147×10^4
5	72	1.75×10^4	$.47 \times 10^4$– 6.5×10^4
Control	423	1.37×10^5	$.44 \times 10^5$– 4.32×10^5

* No. of Salmonella required to infect approximately 50% of the mice.

¶ For assistance in the statistical treatment of the data, the authors are indebted to K. A. Brownlee of the Committee on Statistics, The University of Chicago.

or 4 days after inoculation.

That the successful establishment of Salmonella in the intestinal tract represented a genuine infection was confirmed by the demonstration of visceral involvement in mice killed for culture. Of those with Salmonella still present in their feces, 90% had the inoculated strain in their spleens and 72% in heart's blood as well.

Smaller doses of streptomycin—10, 7 or 5 mg—were less effective than 50 mg, and 1 mg caused no increase in susceptibility.

The effect produced by 50 mg streptomycin did not persist. Susceptibility to infection diminished but was still detectable on the 5th day.

The mice used seemed to be particularly suitable for experiments designed to demonstrate increased susceptibility to Salmonella infection since they possessed a considerable degree of (natural or acquired) resistance to this microorganism. Despite a high carrier rate, they seldom showed diarrhea or other signs of Salmonellosis, even though they were often held in stock for several weeks before use. Moreover, the LD_{50} (21 days) by intraperitoneal inoculation was 10^6 for the strain of Salmonella used in these experiments. Their resistance to Salmonella infection was further demonstrated by the very low mortality during the period of observation (usually 2 weeks). Only 10 mice died in all experiments although bacteremia was a common occurrence in the infected mice.

No attempt was made to recover from feces any but the inoculated streptomycin-resistant strain of Salmonella. However, from heart's blood and spleen, which were always cultured on both streptomycin-containing and streptomycin-free media, streptomycin-sensitive Salmonella were recovered from about 6% of the animals autopsied. Most of these strains belonged to group D, which includes *S. enteritidis,* and a few to group B, which includes *S. typhimurium.* These naturally occurring (streptomycin-sensitive) Salmonellae have been disregarded in the tabulated data in which positive cultures refer only to the streptomycin-resistant strain used for inoculation.

The increased susceptibility of the mouse's intestinal tract to infection after treatment with streptomycin is believed to have been caused by changes in the enteric flora resulting from the antibacterial action of the drug rather than to any stimulating effect on the inoculated organism or toxic effect on the host.

To be sure, Welch, Price and Randall(11) observed that very small doses (.25-1.0 mg) of streptomycin increased the mortality of mice infected intraperitoneally with *S. typhosa,* and Jackson and Axelrood(12) have recently reported that the mortality of mice infected intraperitoneally with *Pseudomonas aeruginosa* was somewhat increased by subcutaneous treatment with 1 mg Chlortetracycline. They observed no such effect, however, in mice infected intraperitoneally with *Proteus vulgaris* or with a drug-resistant staphylococcus, although among the latter there was some increase in the severity of bacteremia 2 to 3 hours after inoculation.

In vitro stimulation of bacterial growth in the presence of subinhibitory concentrations of various antibiotics has been described by a number of workers(13). No evidence was obtained, however, that the growth of the strain used in these experiments was stimulated by streptomycin either *in vitro* or *in vivo.* Nor was any toxic effect apparent in mice treated by mouth with even larger doses of streptomycin. This was not surprising since little if any streptomycin is absorbed from the gastrointestinal tract(14). It is for these reasons that the effect of streptomycin herein described is attributed not to its action on the inoculated strain of Salmonella, nor on the tissues of the mouse, but rather to its antibacterial effect on the microflora within the intestinal tract. The nature of the changes in the microflora responsible for the increased susceptibility to infection are being investigated.

It seems possible that this method for increasing the susceptibility of a laboratory animal to infection by direct inoculation into the gastrointestinal tract may prove useful in the experimental study of other enteric infections.

Summary. 1. Preliminary treatment by mouth with a large dose (50 mg) of streptomycin increased the susceptibility of mice to infection following oral inoculation with a

streptomycin-resistant strain of *Salmonella enteritidis*. In mice treated with streptomycin 24 hours before inoculation, <3 Salmonella sufficed to initiate infection in 50% as compared with approximately 10^5 in untreated controls. 2. This effect of streptomycin decreased as the interval between treatment and inoculation was lengthened but was still detectable on the 5th day. Smaller doses of streptomycin (5-10 mg) resulted in smaller increases in susceptibility. 1 mg was ineffective. 3. Representative numbers of mice killed for culture showed the spleen to be infected in 90% and heart's blood in 72% of those with positive fecal cultures at the time of autopsy. 4. It is believed that this increase in susceptibility following streptomycin treatment resulted from a disturbance of the normal intestinal microflora caused by the antibacterial action of the drug. 5. It is suggested that this method may be applicable to the experimental study of other enteric infections.

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In vitro Conversion of Thyroxin to Triiodothyronine by Kidney Slices.* (21031)

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(Introduced by O. O. Meyer.)

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Several reported observations suggest that 3-5-3'L-triiodothyronine is derived from thyroxin by deiodination in extrathyroidal tissues (1-3). This concept is supported by the following observations on kidney slices, demonstrating *in vitro* deiodination of thyroxine to triiodothyronine.

Methods. 150-200 g male rats (Sprague-Dawley strain) were used. The animals were sacrificed by decapitation, the kidneys aseptically removed and cut into thin slices. Slices representing approximately one-half of a kidney were placed in each of the Warburg flasks containing 3 ml of Krebs-Ringer phosphate solution supplemented with 0.03 mg glucose. 0.01 μg of chromatographically pure I^{131} labelled thyroxin† were added to each flask. The slices were incubated at 37°C for periods of 0, 3, 6, 9, and 12 hours. Controls were run as follows: To one flask incubated for 12 hours potassium cyanide was added to yield a final concentration of 0.01 M; to a second flask incubated 6 hours potassium iodide was added to yield a final concentration of 0.01 M; in a third flask incubated 12 hours kidney slices which had been boiled for 5 minutes were used; to a fourth flask incubated 12

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† Obtained from Abbott Laboratories.

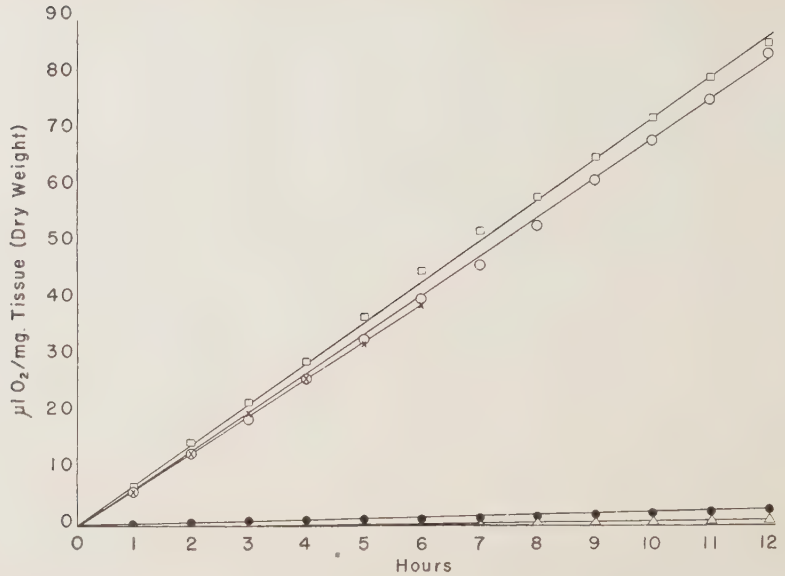

FIG. 1. Oxygen uptake by kidney slices. ○———○ represents untreated tissue; □———□ antibiotic control; ×———× potassium iodide control; ●———● potassium cyanide control; △———△ boiled control.

hours, 1000 units of penicillin G and 100 μg of streptomycin were added. At the end of the incubation period the slices were removed from the media, dried with blotting paper, weighed and washed with water to remove excess radiothyroxin. They were then homogenized in 2 ml of distilled water. The pH of the homogenates was adjusted to 3.0 by the addition of 0.1 N hydrochloric acid. The radioactivity of the entire homogenate was

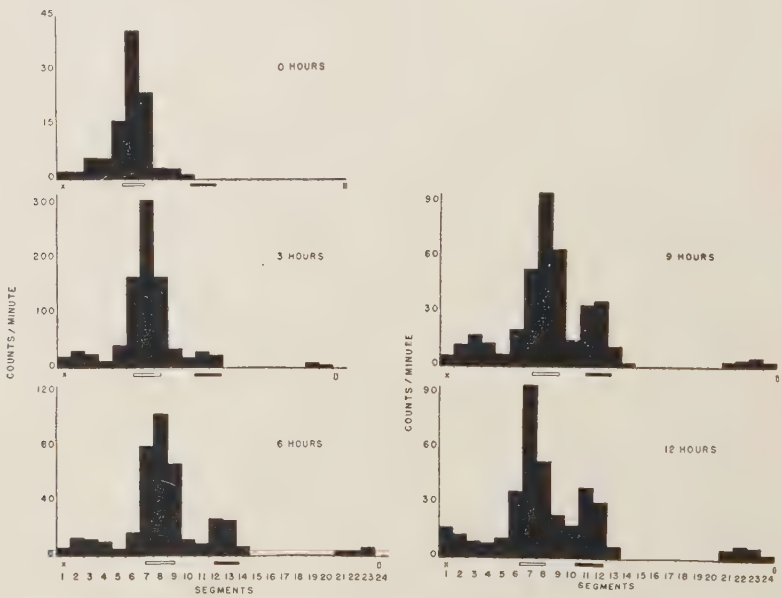

FIG. 2. Distribution of radioactivity in chromatograms following varying periods of incubation. The starting point of chromatography is indicated by ×, the developer front by the vertical bar. The position of thyroxin is shown by the horizontal open bar, that of triiodothyronine by the horizontal closed bar.

determined initially and following each extraction procedure by counting in a well-type scintillation detector. Extraction of the homogenates was carried out with 6 ml portions of water-saturated butanol. Five extractions were necessary for the removal of approximately 95% of the radioactivity. The butanol extracts were pooled and evaporated to dryness at room temperature. The residue was dissolved in 0.1 ml of butanol containing 50 μg of carrier L-triiodothyronine.‡ The final solution was subjected to single dimension chromatography as previously described (4). The chromatograms were cut transversely into 0.5 cm numbered segments and counted in a bell-type end window Geiger counter. After counting, the segments were reassembled and the color of the carrier triiodothyronine was developed as previously reported(4). Coincidence of the radioactivity with the carrier triiodothyronine spot was taken as an indication of conversion of labelled thyroxin to triiodothyronine.

Results. Fig. 1 shows the oxygen uptake of slices, indicating functioning tissue throughout the duration of the experiment. Fig. 2 shows the distribution of radioactivity in the chromatograms following various periods of incubation. The initial peak represents radioactive thyroxin and the second peak corresponds to triiodothyronine. Significant radioactivity begins to appear in the triiodothyronine spot at 3 hours. Increasingly greater concentrations are found at 6, 9, and 12 hours. In Fig. 3 is shown the effect of the addition of potassium iodide, potassium cyanide, antibiotics, and of boiling on the conversion of thyroxin to triiodothyronine. It is clear that the conversion is reduced in the presence of potassium iodide and is abolished in the presence of 0.01 M cyanide or by boiling. The presence of antibiotics did not affect the conversion. The radioactivity localized behind the developer front has not been identified, but is known not to be iodide, which has an Rf value less than that of thyroxin.

Discussion. The demonstration of 3-5-3′L-triiodothyronine in the thyroid gland and in plasma(5-8) and the subsequent observations that it has several times the physiological activity of thyroxin(2,9,10) has led to speculation that it is the peripherally active form of the thyroid hormone. It is evident, however, that thyroxin is present in plasma in

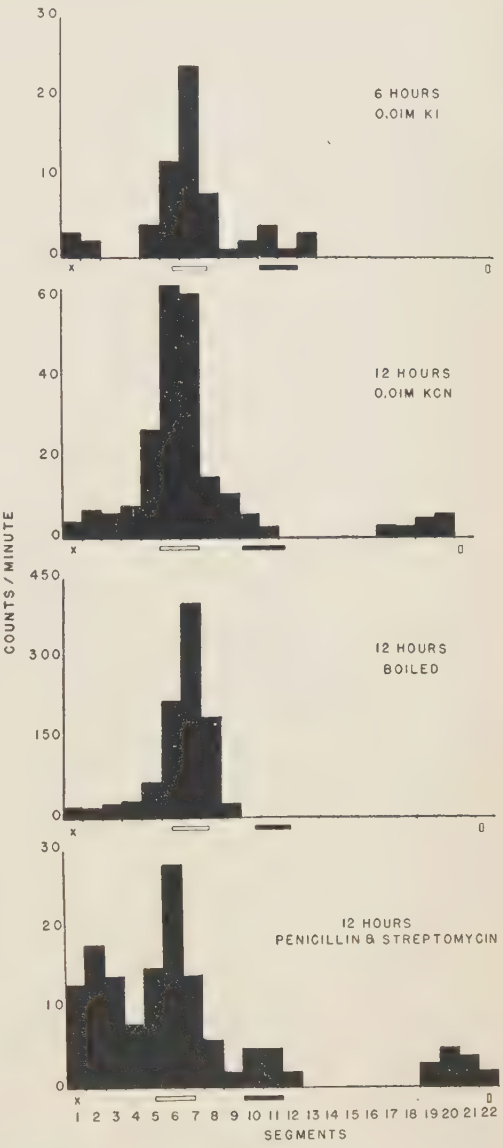

FIG. 3. Distribution of radioactivity in chromatograms of controls. The starting point of chromatography is indicated by ×, the developer front by the vertical bar. The position of thyroxin is shown by the horizontal open bar, that of triiodothyronine by the horizontal closed bar.

‡ Triiodothyronine was kindly supplied by Dr. Arthur Heming of Smith, Kline & French Laboratories.

greater quantities than triiodothyronine(11-13). These observations have led to the suggestion that thyroxin is converted peripherally to triiodothyronine by a process of deiodination. In support of this possibility, Gross and Leblond found a compound, later identified as triiodothyronine, in the organs of thyroidectomized mice following the administration of I^{131} labelled thyroxin(1). These observations have been confirmed recently by Kalant *et al.* in propyl thiouracil-treated animals(3).

The data reported in this paper demonstrate that extrathyroidal conversion of thyroxin to triiodothyronine can occur, at least in the kidney.

Summary and conclusions. 1. Chromatographically pure I^{131} labelled l-thyroxin was added to surviving kidney slices which were incubated in a modified Krebs-Ringer phosphate solution. 2. Following incubation, the slices were homogenized, extracted with butanol and the thyroxin and triiodothyronine separated and identified by paper chromatography. 3. The triiodothyronine was found to be radioactive. This was taken as evidence of deiodination of thyroxin to triiodothyronine. 4. Conversion of thyroxin to triiodothyronine was reduced by excess iodide and abolished by boiling the tissue or by the addition of potassium cyanide to the media.

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Distribution and Properties of an Unidentified Growth Factor for Avian *Lactobacillus bifidus.** (21032)

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Veltre, Shorb, and Pelczar(1) reported that *Lactobacillus bifidus* of avian origin failed to grow in a defined medium unless supplied with a substance(s) found in Phytone, yeast extract, beef extract, or fish solubles. A disaccharide from mucin, galactose acetylglucosamine(2,3), and blood group substances A and B(4) failed to replace the requirement of the avian bifids as these compounds did for the variants of *L. bifidus* from humans. Because large numbers of the *L. bifidus* organisms were associated with a depression of chick growth(5) perhaps by robbing the avian host of some required nutrient, further studies on the distribution and properties of the substance required by these avian bifid organisms have been carried out.

Experimental. An assay procedure has

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TABLE I. Composition of Basal Medium.

	Amt/500 ml double strength medium
Cas amino acids	5 g
KH_2PO_4	2.5
Lactose	7
Sodium acetate (anhydrous)	5
Adenine, guanine, uracil, xanthine	10 mg each
$MgSO_4 \cdot xH_2O$	200 mg
$NaCl$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$	10 mg each
Ascorbic acid	2 g
Cysteine HCl	200 mg
Asparagine	100
p-Aminobenzoic acid	10 μg.
Pteroylglutamic acid	10
Thiamine · HCl	200
Ca pantothenate	400
Nicotinic acid	600
Riboflavin	200
Pyridoxine HCl	1200
Biotin	4
Distilled water	500 ml
pH to 6.8	

been developed for the avian *L. bifidus* factor (ALbf), based on the observations of Veltre *et al.*(1) using poult strain T3 (ATCC 11,617). The composition of the basal medium is shown in Table I. All of the ingredients except the B vitamins and ascorbic acid were mixed and frozen. Just before use, the B vitamins, maintained as 2 stock mixtures(6) and ascorbic acid were added. The medium was adjusted to pH 6.8. The total volume in the 22 x 108 mm tubes was 6 ml, 3 ml being double strength medium and the remaining 3 ml consisting of sample and water. The assay tubes were autoclaved for 5 minutes at 120°C. The stock culture was transferred weekly on basal medium stabs containing supplements of 2% agar and approximately 50 mg of water soluble solids from fish solubles per 6 ml of single strength medium. The inoculum medium was of the same composition, with agar omitted. Because niacin was gradually destroyed by storage of the complete medium, the inoculum medium was made bi-weekly, autoclaved and frozen. It was thawed and warmed just before inoculation. The 24-hour inoculum culture was centrifuged once, and resuspended in physiological saline to give a galvanometer reading of about 85 on the Evelyn colorimeter, using the 620 mμ filter. One drop of inoculum was used per tube. The assay tubes, as well as the stock cultures and inoculum cultures were incubated aerobically. After 66 hours incubation, the assays were read by titrating to pH 7, using 0.05 N NaOH. A hot water extract of fish solubles (Gorton-Pew) was chosen as the standard. All samples were prepared as hot water extracts unless otherwise stated and all unit values were calculated on the basis of water soluble solids. The pH of the samples and standard was adjusted to 6.8 because *L. bifidus* has a very limited pH growth range. An excess of acid or alkali will completely prevent initiation of growth.

Results. A typical standard curve is shown in Fig. 1. The amount of fish solubles solids required for one-half maximum growth, when growth was measured by acid formation, was about 12.5 mg after 60 hours incubation. The assay organism produced rather limited amounts of acid, the highest titration values with optimum amounts of fish solubles being between 7 and 10 ml of 0.05 N acid. Doubling the amount of lactose in the medium did not improve the amount of acid formed. A steeper pitch in the slope of the standard curve was observed with the larger dosages of fish solubles or with some other samples, suggesting that the samples were carrying materials which were fermentable, giving rise to more acid. For calculating potencies of samples, only that part of the curve lying between

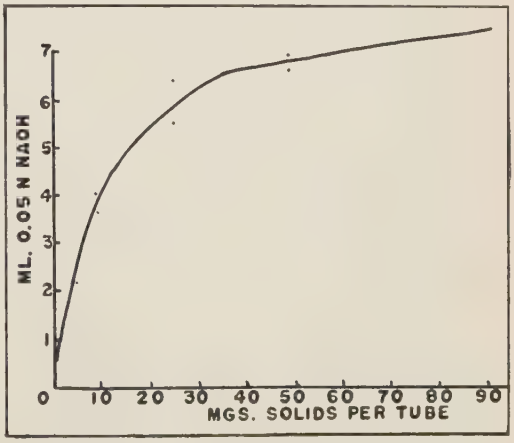

FIG. 1. Dosage response curve of *Lactobacillus bifidus* T3 (ATCC 11,617) with fish solubles standard, after 60 hr incubation.

TABLE II. Activity of Source Materials for Avian *Lactobacillus bifidus* Factor.

Samples (as hot water extracts)	Units*/mg of water soluble solids
Animal products and isolated proteins†	
Fish solubles	1.0
Crabmeal	3.1
Beef extract	1.7
Liver NF2 (Armour)	2.2
Chick heart	2.2
" livers	.3–1.6
" fecal contents (from 7 groups of chicks on casein-gelatin-cerelose diet, various supplements)	3.4–5.5
Plant products and isolated proteins†	
Phytone (Baltimore Biol. Lab.)	1.4
Alfalfa meal	1.6
Tomato pulp	2.5
Grass juice	3.0
Asparagus juice concentrate	3.6
Corn steep liquor	4.2
Fermentation products, bacterial cultures and yeasts†	
Penicillin mycelium residue	2.8
Soludri (Schenley)	2.9
Ethyl molasses solubles	2.8
Escherichia coli, parent strain,‡ 1-day incubation	4.8
Idem, 5-day incubation	1.4
E. coli, methionine-requiring strain,§ 1-day incubation	2.9
Aerobacter aerogenes, chick strain, Koser's citrate culture	2.2
A. aerogenes, poult strain, Koser's citrate culture	4.9
Bacillus subtilis preparations†	.6–20.0
" *megaterium* PYP 25	2.7
Yeasts and yeast products†	1.5–16.0

* 1 unit equivalent to 1 mg of water soluble solids from fish solubles.

† Egg albumin, egg yolk, Hi Pro (hydrolyzed chicken feathers), gelatin, whey, casein, trypsinized casein, trypsin, pepsin, pancreatin, Mylase P, fishmeal, whale solubles, molasses, soybean meal, alpha protein and Dracket protein, contained less than 1 unit/mg water soluble solids. Of the 16 *B. subtilis* cultures and preparations tested, 1466A and 1466B had activities of 20 and 14 units/mg respectively. Of the 14 yeast preparations tested, one preparation of *Candica flarari,* Pabst 186-819D, had 16 units/mg.

‡ Parent strain(7) of *E. coli,* kindly supplied by Dr. Esther Stubblefield, The Upjohn Company. It was grown on Koser's citrate broth, with 0.5% glucose.

§ Grown on methionine assay medium, with methionine added.

2 and 7 ml of acid was used. One mg of water soluble solids from fish solubles was adopted as one unit of ALbf. Day to day reproducibility was rather good with the fish solubles standard but a trial standard prepared from Pabst yeast R_1 was found to lose about half its activity over a 6-week period, during which time the preparation was kept frozen. Fish solubles from different sources and batches varied in ALbf content. Some samples, especially those with rancid odor contained inhibitory substances.

Table II shows the distribution of ALbf in various source materials of animal and plant origin, in fermentation products, yeasts, and bacterial cultures. Isolated proteins, milk products and some animal products have a rather low content of ALbf, less than one unit per mg of solids. Fish products, chick organs, liver NF2, and some plant materials, have slightly more activity, between one and 3 units per mg. The cecal content of chicks, however, had a higher value, between 3.4 and 5.5 units per mg, in the same range of activity as extracts of vegetables and fermentation products, such as corn steep liquor, asparagus juice concentrate, grass juice extract, penicillin mycelium residue, and distillers solubles. The high content of ALbf in products produced by microbial activity suggested that pure cultures of microorganisms might contain the factor. Whole cultures of *Escherichia coli*(7) and *Aerobacter aerogenes,* grown on Koser's citrate-glucose broth, itself inactive, produced from 1.4 to 4.9 units of ALbf per mg. One culture of *Bacillus megaterium* and a series of *Bacillus subtilis* culture preparations contained ALbf in amounts ranging from 0.6 to 20 units per mg. A series of yeasts and yeast products showed activity from 1.5 to 16 units per mg. Materials found to be inactive, in addition to those previously reported(1) were yeast nucleic acid and strepogenin(8), using fraction A(9) which is active for *Lactobacillus bulgaricus* 09. With the exception of whey, the distribution of ALbf showed a similarity of distribution with those materials containing unidentified chick growth factors. The chick growth factors have been reported in whey, fish meal, fish solubles, liver, grass juice, alfalfa, distillers solubles, various yeasts, *A. aerogenes, E. coli,* and *B. subtilis.* The work on the distribution and fractionation of these chick factors has recently been reviewed(10-19). It was therefore of interest to determine whether the factor required by

TABLE III. Activity of Fish Solubles,* Liver† and *Escherichia coli* Fractions for *Lactobacillus bifidus* and Chicks.

Sample	Conc. of *L. bifidus* activity	Conc. of chick activity
Fish solubles	1.0	1‡
Idem, hot water extr.	1.3	
" , fraction M12	1.0	38‡
" , " M26	1.3	190‡
Liver NF2	2.2	1§
Idem, phenol-butanol solvent fraction	6.6	58§
Liver Biopar C, counter current tube 4	.0	340§
Idem, tube 8	1.3	0§
E. coli, whole culture	2.9	Active§
Idem, ethanol eluate from Norit A	.4	" §

* Fractions M12 and M26 kindly supplied by Dr. Henry Menge, Poultry Husbandry Division, Agricultural Research Center, Beltsville, Md.

† Fractions from liver NF2 and Biopar C prepared by Dr. Henry Menge, Dr. G. H. Arscott, and Dr. G. F. Combs, Poultry Husbandry Department, Univ. of Md.

‡ Activity based on organic solids, data supplied by Dr. H. Menge.

§ Data supplied by Dr. H. Menge, Dr. G. F. Combs, Dr. G. H. Arscott and Dr. G. L. Romoser, Poultry Husbandry Department, Univ. of Md.

L. bifidus was identical with any of the chick growth factors. Potent chick fractions were obtained and tested for *L. bifidus* activity.

Table III shows the activity of the chick fractions for *L. bifidus* and for the chick. The chick activity has been expressed in terms of concentration during fractionation. Fraction M12 from fish solubles showed increased activity for the chick but not for *L. bifidus*. Fraction M26, very active for the chick, had low activity for *L. bifidus*. A series of fractions from liver NF2 and liver Biopar C were also tested. Liver NF2 was somewhat more active than fish solubles for *L. bifidus*. *L. bifidus* activity followed, to some extent, the chick activity through the phenol-butanol solvent procedure, but it did not follow the chick activity after counter current distribution. The chick activity was concentrated in tube 4, which was inactive for *L. bifidus,* while tube 8 contained some *L. bifidus* activity but was inactive for the chick. An *E. coli* culture and the alcoholic eluate from a Norit treated culture of *E. coli,* both having activity for the chick(20), had some *L. bifidus* activity, although the eluate had lower ALbf content than the original culture. Whey had low ALbf content and no fractions prepared from whey increased *L. bifidus* growth. No concentrates of the grass juice or alfalfa chick factors have been available for testing. The distribution of ALbf coincides with the distribution of these factors in some products.

The possibility existed that fractionation of the chick growth factors might destroy a part of ALbf, if ALbf were multiple in nature. Therefore various inactive substances such as extra casein, egg yolk, egg albumin, vitamins, and growth factor supplements have been tested with M12 or similar ALbf-inactive chick-active fractions, without producing growth of *L. bifidus*. Galactose-acetylglucosamine added to various ALbf-inactive chick-active fractions did not result in growth of *L. bifidus*. Growth was depressed slightly when the disaccharide was added to active ALbf fractions. Before embarking on a fractionation program it was of interest to determine some of the chemical and physical properties of the factor using fish solubles as the source material. The activity was soluble in phenol or water and in laboratory grade methanol, tertiary butanol, and ethylene glycol monomethyl ether. It was soluble in ethanol-water and ethylene glycol monobutyl-water solvent systems, but not soluble in petroleum ether, ethyl ether, absolute ethanol, acetone, chloroform, carbon tetrachloride, toluene, n-butanol, benzene, pyridine, ethylene glycol monobutyl ether, dioxane, caprylic alcohol or benzyl alcohol. It was absorbed on supercel, Norit A and alumina. It was eluted from Norit A by phenol, methanol-water, ethanol-water, and the cellosolve water systems. The activity was not eluted from alumina by any of the solvents tested. Combinations of alumina eluates were not active. The factor acted as a cation on paper electrophoresis and it was dialyzable. The growth factor, contained in a phenolic eluate from Norit A, moved as one elongated unit on paper chromatograms in a 60% ethylene glycol monobutyl ether-water solvent system, the Rf value being between 0.4 and 0.7. A rechromatographed sample lost activity completely, suggesting separation of factors or destruction of activity. The activity disappeared on treatment with potassium perman-

ganate, lead acetate and silver nitrate. All the fractions from these last 3 treatments contained inhibitors, in a plate assay, so that activity could be masked by toxic substances. In this respect, phenol fractions also contained inhibitors. ALbf activity was not destroyed by autoclaving at either pH 0.9 or pH 10 for 30 minutes at 15 lb pressure, by standing from 30 minutes to 3 hours at room temperature with 6 N HCl, nor by exposure to ultraviolet light for one hour. It was partially destroyed by 15 minutes exposure to concentrated nitric acid at room temperature, by autoclaving at 15 lb pressure for 30 minutes at pH 12 and by exposure to sunlight for one week. The activity was completely destroyed by refluxing for one hour with 6 N HCl. Ash from fish solubles was inactive.

Various procedures have been tried in concentrating the growth factor. Approximately 6- to 10-fold increases in activity have been obtained in 70% ethanol or 60% ethylene glycol monobutyl ether eluates from Norited water extracts of yeast R_1 and fish solubles. No concentration was obtained in the isobutyl alcohol precipitates of fish solubles by the method described by Weise *et al.*(21) for the chick growth factor in fish solubles. The precipitate was inactive for *L. bifidus* and the activity remained in the water layer of the 80% isobutyl alcohol-water mixture. Further work is in progress on fractionation of the *L. bifidus* factor.

Two branched bifids of human origin (ATCC 11,146 and 11,147) and 2 unbranched strains of human origin (ATCC 4962 and 4963) originally classified as *L. bifidus* but now classified as *L. acidophilus,* have been used as assay organisms in the ALbf assay. None of these cultures would grow with yeast R_1 at 2.85 mg per 6 ml, the amount causing optimum growth of the avian bifids. Perhaps these cultures would grow in the presence of galactose acetylglucosamine, the disaccharide from mucin, but this was not tested. The mutants of human *L. bifidus* requiring this disaccharide(2,3) were not available for use in our assay.

Discussion. The avian *L. bifidus* factor is not identical with that factor reported by György and associates(22-25) in human milk, which promotes the growth of human *L. bifidus* variants. The substances which influence the growth of the human variants, galactose acetylglucosamine(2,3) blood groups substances A and B(4) as well as pantethine(3) and ammonic compounds(4) were inactive as ALbf. The distribution of the 2 factors is entirely different, yeast extract and vegetable extracts being inactive for the human bifids(3,22) while very active as ALbf.

Although no positive identity has been established between ALbf and the liver, whey, *E. coli, B. subtilis* or fish solubles chick growth concentrates, the common distribution of factors suggests that further work may establish some relationship. Lack of correlation is not surprising in view of the recent unpublished work in this department(26) and elsewhere (27) indicating that 2 or more factors are acting together to influence chick growth. The low content of ALbf in casein, gelatin and Dracket protein would suggest that these ingredients might be used in a basal diet in developing a chick assay for *L. bifidus* factor. Such tests are now being tried in this department. Success in such tests may depend on the amount of intestinal synthesis of ALbf, because cecal contents have been demonstrated to be potent sources of the ALbf activity.

Summary. A method of assay for a growth factor required by *L. bifidus* of avian origin has been developed. The factor is found in low amounts in isolated proteins and animal products, in higher amounts in plant materials, and in highest amounts in fermentation products, yeasts and bacterial cultures. The growth factor was not found in concentrates of liver, whey and fish solubles which were potent sources of unidentified chick growth factors. Chemical and physical properties of the *L. bifidus* factor are described.

We are indebted to Dr. J. C. Lewis, Western Regional Research Laboratory, U. S. Department of Agriculture, for the *B. subtilis* and *B. megaterium* preparations; to Dr. Alexander Frieden, Pabst Laboratories for the Pabst yeast samples; and to Dr. R. M. Tomarelli, Wyeth Laboratories, Inc. for the disaccharide from mucin.

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Effect of β,β-Diethylalanine on Composition of Rat Livers. (21033)

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The effects of oral and intraperitoneal administration of β,β-diethylalanine (DEA) on growth and liver composition in rats has been studied with the view that it might be an antagonist of valine, isoleucine, or leucine. An antagonism involving one of these amino acids would be particularly advantageous for studying protein synthesis because, unlike methionine and tryosine, these amino acids are not known to be concerned with anabolic reactions aside from those occurring in the synthesis of protein molecules.

Materials and methods. Synthesis of DL-DEA. A modified malonic ester synthesis has previously been employed for the preparation of DL-DEA(1). The material for these experiments, however, was synthesized by HCl hydrolysis of the corresponding hydantoin (m.p. 182°) prepared by the general hydantoin synthesis of Bucherer *et al.*(2,3). The HCl was removed on an amberlite IR4-B ion exchange column. An overall yield of 41% was obtained. The product was identified by synthesis of the chloracetyl derivative(1) which gave the theoretical neutralization equivalent [m.p. 130°; DuVigneaud *et al.*(1) 127-128°]. On paper chromatograms the free amino acid gave a single ninhydrin positive spot. *Feeding of DL-DEA.* The control diet A contained vitamin-free casein (Labco), 10;

sucrose, 30; corn starch, 34.9; salts(4), 2.5; corn oil (Mazola), 15; cod liver oil, 3; Ruffex, 2; vitamin mix(5), 1; choline chloride, 0.1; L-methionine, 0.4; and L-glutamic acid, 1.10%. In diet B, 1.09% DL-DEA replaced 0.55% L-glutamic acid and 0.54% corn starch of the control diet. In diet C, 2.17% DL-DEA replaced 1.10% L-glutamic acid and 1.07% corn starch of the control diet. Twenty-four male white rats,* housed in individual cages, were placed on the control diet (diet A) for 4 days preceding the growth studies. After this period of adjustment to the synthetic diet, they were divided into 3 groups of 8 rats (groups A-C). Each group is designated by the letter of the diet on which it was maintained. *Ad libitum* food consumption was recorded every 2 days and body weights every 4 days. After 28 days on the experimental diets, the rats were sacrificed and the livers analyzed for lipide, water, and protein content (TCA insoluble material) as previously described(5). *Injection of DL-DEA.* In those experiments in which DEA was administered in large doses, 160-200 g rats were fasted for 12 hours prior to the first injection. Four intraperitoneal injections of 100 mg of DL-DEA in 1.0 ml of 0.9% saline were given at 2.5 hour intervals. The fasting was continued for 36 hours after the first injection, at the end of which time the rats were sacrificed and the livers analyzed as in the feeding experiments. Four female rats, 2 from each of 2 litters, received DEA injections while 4 female rats, 2 from each of the same 2 litters, served as controls. The controls were treated in a similar manner but received only saline solutions. The experiments were repeated on a similar grouping of 8 male rats. The rats were allowed fresh water throughout the experiment.

Results. Preliminary feeding experiments had indicated that DEA stimulated growth to a slight extent when substituted for corn starch in a basal diet. Diets A, B, and C were subsequently chosen so that each would contain the same amount of available nitrogen on the assumption that one-half of the DL-DEA nitrogen was utilizable. On these diets

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TABLE I. Effects of Feeding DL-β, β-Diethylalanine on Growth of Male Rats.

Group	Avg initial wt, g	Avg wt at 8 days, g	Avg wt at 28 days, g	Gain in wt/g food consumed (g) 1-8 days	9-28 days
A	74	107	176	.39	.32
B	73	84	156	.19	.32
C	71	85	154	.24	.31

the growth of groups B and C (Table I) was nearly identical for the 28-day period, but both groups showed initial growth lags when compared with group A, due at least in part to lowered food consumption. While it is possible that in both groups B and C the lower lipide content (Table II) is a reflection of the poorer growth of these groups during the first 8 days on the new diet, it seems likely that the last 20 days of parallel growth and nearly identical growth response per gram of food consumption of all groups would have overcome any such initial effect. It is, perhaps, more reasonable to suppose that DEA acts specifically to decrease the lipide content but that it has reached almost its maximum effectiveness at the lower level (diet B). The effect of feeding DEA upon the protein content (Table II) of the livers cannot be explained satisfactorily by the initial growth lag shown by groups B and C since the protein content was significantly higher only in group C on the higher level of DEA.

The injections of DL-DEA produced no significant differences in liver lipide, protein, or water content as compared with controls. The average liver lipide contents expressed as per cent of body weight for the male and female experimental and control groups ranged from 0.22 to 0.24. The average water content of the livers ranged from 70.1 to

TABLE II. Effect of Feeding DL-β, β-Diethylalanine on Liver Lipide, Water, and Protein.

Group	Total liver lipide of body wt, %	Water content of liver, %	Liver protein of body wt, %
A	.51 ± .05*	66.5 ± .5	.610 ± .010
B	.34 ± .04	68.3 ± .6	.622 ± .010
C	.31 ± .02	68.8 ± .2	.657 ± .008†

* Stand. error of mean.
† One sample lost.

71.6% for the same groups. Expressed as per cent of body weight, the liver protein contents of the female DEA injected rats and female control rats were 0.74 and 0.71 respectively, while the corresponding values for the male DEA injected rats and male controls were 0.80 and 0.81 respectively.

Although DEA was not a growth inhibitor on the levels at which it was administered, the differences in liver composition of DEA fed animals and controls are of interest, particularly in view of similar experiments with ethionine(5). The fact that feeding either DEA or ethionine can result in higher water and protein content of livers as compared with controls suggests that these effects are due to their properties as amino acid analogues *per se,* and not to interference with specific functions of the amino acids involved (*e.g.* transmethylation). Since the effect of DEA is to lower and the effect of ethionine is to raise the level of liver lipide(5), it appears likely that the mechanisms of action of these compounds on lipide metabolism are different. The scope of these experiments is not sufficient to suggest the mechanism by which the DEA diet lowers the liver lipide content.

Summary. The effect of the administration of β,β-diethylalanine (DEA) upon the composition of rat livers has been studied. Feeding DEA to male rats resulted, at the higher of 2 levels, in lower liver lipide and higher liver water and protein content than in controls. Injections of massive doses of DEA were without significant effect. The implications of these results with respect to similar studies with ethionine are discussed.

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Effect of Aldosterone and Desoxycorticosterone on Adrenalectomized Dogs.* (21034)

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Since 1950, data have rapidly accumulated concerning the presence in adrenal cortical extracts and in adrenal vein blood, of a highly active Na-retaining substance(1-6). This work has culminated in recent announcements from 3 laboratories of the isolation in crystalline form of a new mineralcorticoid(3,7,8), provisionally designated by Simpson *et al.*(7) as electrocortin. Chemical degradation of the new crystalline adrenal steroid by Simpson, Tait, Wettstein, Neher, Euw, Schindler and Reichstein(9) shows that the compound is 11β-21-dihydroxy-3,20-diketo-4-pregnene-18-al. According to these investigators, this reacts mainly as the 11-hemiacetal when in solution; they suggested aldosterone as a definitive name for the compound. Owing to scarcity of material, few detailed physiological studies on the pure substance have appeared (10-12).

Through the courtesy of Professor T. Reichstein, the writers secured enough crystalline material for detailed tests on 4 adrenalectomized dogs. The present study is a comparison of the life maintenance and Na-retaining activity of pure crystalline aldosterone with that of the free alcohol of desoxycorticosterone (DOC).

* Expenses of this investigation were defrayed by grants from Sharp and Dohme Division of Merck & Co., and the National Science Foundation. DOC was supplied by Ciba Pharmaceutical Products, and the aldosterone by Professor T. Reichstein of the University of Basle, Switzerland.

Material and methods. Earlier work(13), showed that 10% aqueous-alcohol is an excellent vehicle in which to administer the free alcohol of adrenal steroids when rapid absorption is necessary. This vehicle also appears to be superior to oil or water preparations or micro-crystalline suspensions for life maintenance studies. Therefore, the aldosterone and DOC crystals were dissolved in sufficient 95% ethanol which when diluted to 30% with distilled H_2O yielded a solution containing 25 μg/cc of aldosterone and 1.5 mg/cc of DOC. This constituted the stock solution which if kept in sterile bottles can be left at room temperature for long periods without deterioration. Each day the dosage to be used was removed from the stock solution, diluted to 10% alcohol and administered subcutaneously in divided doses, one-half at 9:30 a.m. and the remainder at 4:30 p.m. Four dogs were used for testing aldosterone, 3 for DOC, and 4 each for assay of cortisone and hydrocortisone. In this brief report, however, but 2 representative experiments (one each for aldosterone and DOC) will be discussed. The dog given DOC had been adrenalectomized 1 year and weighed 17 kg; the aldosterone animal weighed 20 kg and the adrenals had been removed 3 years previously. Both animals, prior to these experiments had been employed in bioassay work and studied through numerous cycles of adrenal insufficiency. During the intervals between the various experiments they were maintained in good health on 0.5 mg DCA in oil injected intramuscularly once daily. The diet used in these and earlier experiments has been described(14); however each dog's daily ration contained a total of 1.47 g of Na and 0.94 g of K. This is a low sodium diet for an adrenalectomized dog. The animals were kept at each dose level for 10 days and at the termination of this interval blood samples were taken, the arterial pressure determined and the dosage reduced by 50%. This procedure was followed until subminimum dose levels were reached and signs of adrenal insufficiency appeared.

Results. The essential data regarding the physiological activity of aldosterone and DOC are given in Fig. 1. It is obvious that both steroids, in very small doses, when administered in 10% alcohol are capable of maintaining a normal serum electrolyte pattern, arterial pressure and blood urea nitrogen. The minimum maintenance dose of aldosterone for the dog whose analyses are represented in Fig. 1, is 6.25 μg/dog/day, whereas it required 250 μg of DOC/dog/day to maintain the animal in equally good health. The dogs retained full activity, vigor and appetite at these dosages. Upon reducing the daily amount of aldosterone by one half, or to 3.125 μg/dog/day, the arterial pressure declined, serum K increased as did also the blood urea nitrogen. However, serum Na exhibited little, if any, change. The appearance, appetite and activity of the dogs seemed unimpaired throughout the 10-day interval of this low dose level. Further reduction of dosage to 1.56 μg/dog/day led to development of symptoms of adrenal insufficiency such as lassitude, weakness and spasticity of the hind limbs. Serum K generally increased to the level at which early signs of hyperpotassemia became evident. The arterial pressure slowly declined and blood urea nitrogen became elevated. None of the dogs receiving aldosterone completed the 10-day test period at this lowest dose level.

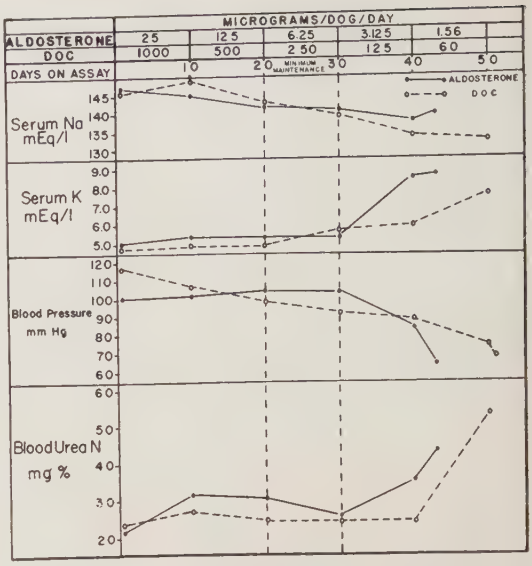

FIG. 1. Effect of aldosterone and DOC on arterial pressure, serum electrolytes and blood urea nitrogen of adrenalectomized dogs.

Thus, the minimum maintenance requirement for this aldosterone-treated dog is 6.25

μg/dog/day, the subminimum dose is 3.125 μg/dog/day, and the level at which substitution therapy is necessary is 1.56 μg/dog/day. However, when the data obtained from study of all 4 animals that received this steroid are analyzed, individual variations appear. In all cases the minimum dose found adequate to maintain normal serum electrolytes, arterial pressure, blood urea nitrogen and body weight, was approximately 10 μg/dog/day. The 3 animals receiving DOC exhibited some variation with respect to their maintenance requirement for the steroid, which was 250-125 μg/dog/day. Thus, aldosterone appears to be about 25 times more potent than DOC under these experimental conditions. These data are in agreement with those of others (7-10). However, our data show that a larger daily dose is required to prevent a slow decline in arterial pressure than is necessary to maintain a normal serum electrolyte pattern. Excluding this slow decline in blood pressure, the minimum daily dose of aldosterone is 6.25 μg/dog/day and for DOC, the dose required varied between 250-125 μg per day with 2 of 3 dogs requiring the larger dose.

Earlier observations(6) by the writers, employing a refined (maximum 60% purity), Na-retaining preparation of the amorphous fraction of adrenal extracts given to us for testing by Dr. E. C. Kendall of this University, had shown that it was not uncommon for adrenalectomized dogs receiving the Na-retaining material, to develop severe symptoms of adrenal insufficiency despite retention of normal levels of serum Na. It was also noted that hyperpotassemia accompanied by severe cardiac symptoms developed even though the serum Na remained normal. Similar changes may become manifest in the lower dose ranges of aldosterone, *e.g.*, 1.56 μg/day. It is clear from the data in Fig. 1 that the steroid in low dosage is not as effective in maintaining a normal level of serum K as it is in keeping the serum Na constant.

Discussion. Isolation of aldosterone and the observation, made by several groups of investigators that it is much more potent than desoxycorticosterone in its Na-retaining properties, raises the question whether the new steroid differs qualitatively as well as quantitatively from DOC compounds. A ready answer is not now forthcoming owing to the paucity of material available for physiological study; however, certain facts seem to be definitely established. Pure aldosterone is about 25-30 times more active than either the free alcohol of desoxycorticosterone in 10% alcohol or its acetate in oil, when tested for Na-retention and life maintenance on adrenalectomized dogs(7-10). According to some investigators(10), the steroid differs in several notable respects from DOC: 1) it is far superior in retaining Na; 2) its activity in inducing K excretion is only about 5 times greater, and finally, 3) aldosterone has no demonstrable effect on water excretion. Knauff *et al.*(5), state that their partially purified material had no effect on liver glycogen deposition and also gave negative results when tested for reduction of circulating blood eosinophils. On the other hand, Schuler, Desaulles and Meier(12), found aldosterone to be about 30 times as active as free desoxycorticosterone in increasing the liver glycogen of mice, but it was somewhat less active than cortisone and corticosterone in this respect. Gaunt *et al.*(15), found the steroid to have an activity equal to or even greater than cortisone when used on rats in the cold stress test and to be potent, though less so than cortisone in the eosinophil depletion test. These data strongly suggest that although aldosterone exhibits activity resembling that of desoxycorticosterone in certain important respects, it also appears to possess a number of other physiological properties which are qualitatively different from those of DOC compounds.

Summary. The minimum daily dose of aldosterone when administered in 10% alcohol to adrenalectomized dogs is approximately 10 μg/dog/day; the dose of the free alcohol of desoxycorticosterone required is 250-125 μg/day. Thus, aldosterone is about 25 times more active than DOC. The new steroid in low dosage is less efficient in regulating the serum K level than it is in maintaining a normal level of serum Na. Hyperpotassemia and symptoms of adrenal failure may be present in dogs presenting normal levels of serum Na. A larger dose of this mineralocorticoid is required to

maintain a normal level of arterial pressure than is necessary to maintain a normal serum electrolyte pattern. Aldosterone appears to possess certain physiological properties which distinguish it from desoxycorticosterone compounds.

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Relationship between Plasma Mucoproteins and Protein Sugar in Patients with Rheumatoid Arthritis Receiving Cortisone.* (21035)

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It has been shown by Winzler *et al.*(1) that serum mucoprotein concentrations are significantly increased in patients with rheumatoid arthritis. Shetlar and associates(2), and Kelley(3) have demonstrated that in the same disease there is a considerable rise in the carbohydrate content of the total serum protein. Serum mucoproteins are comparatively rich in polysaccharides since they contain approximately 14.5 mg % of carbohydrate(3), whereas in normal sera the total globulin fraction contains only 1.5 to 3.9 mg % of carbohydrate and the albumin fraction, about 1.2 mg %(4). Thus, although mucoproteins represent approximately 0.7% of the total serum protein, they contain about 10% of the total protein sugar.

The relationship between the concentrations of serum mucoprotein and total protein sugar has been studied in the present report in order to test the hypothesis that variations of mucoproteins and total protein sugar in inflammatory diseases may depend upon a common regulating factor.

Methods. Mucoproteins were estimated by the Winzler method and are expressed as mg of tyrosine per 100 ml of original serum (Tyrosine Index). Total protein sugar was determined by the tryptophan method of Shetlar as modified by Badin, Jackson, and Schubert(5). Error in duplicate analyses averaged ±0.3 mg for the Tyrosine Index.

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TABLE I.
Variation of Mucoprotein Concentration of Serum with Total Protein Sugar Concentration.

No. of analyses	Total protein sugar mg % serum Range	Avg	Mucoprotein Tyrosine	Sugar	Mucoprotein sugar × 100 / Total protein sugar	Tyrosine index of mucoprotein (% of cases) <3.5	3.5–4.5	>4.5
			(mg % serum)					
30	90–120	109.0	3.74	13.2	12.1	66.6	13.3	20.1
34	121–140	132.7	4.56	15.9	11.9	29.4	17.7	52.9
20	141–160	148.9	4.59	16.2	10.8	20.0	20.0	60.0
27	161–180	170.7	5.63	19.7	11.6	3.7	14.9	81.4
14	181–200	193.0	6.07	21.2	10.9	0	7.5	92.5
13	>200	235.0	6.60	23.3	10.0			

This is an absolute error which holds over the entire range of 2.5 to 9 mg of tyrosine. The error in carbohydrate determination was found to be ±10% in a range of 90 to 330 mg carbohydrate per 100 ml of serum. One hundred thirty-eight samples were analyzed. They were obtained before, during and after administration of therapeutic doses of cortisone. The sugar bound to mucoproteins was calculated according to the data of Winzler and Smythe(6), and of Levy and Jackson(7). The ratio, sugar/tyrosine, given by these authors varies between 3.85 and 3.26. An average value of 3.5 was chosen as a factor for the calculation of the carbohydrate content from the Tyrosine Index.

Results. The correlation coefficient between the concentrations of serum mucoprotein and the total protein sugar was calculated for the entire group of 138 analyses, and found to be 0.62. Mucoprotein concentration and total protein sugar were compared, the analyses having been grouped according to total protein sugar concentration. No attempt has been made to differentiate those values obtained during cortisone treatment from those prior to or after cortisone administration.

Table I shows that mucoprotein sugar concentration rises with increase in the concentration of the total protein sugar. Mucoprotein sugar varied between 10.0 and 12.1% of the total protein sugar.

In order to study statistically the discrepancies between the 2 tests, the analyses of total protein sugar have been divided into 5 concentration ranges. In each of these was calculated the percentage of cases where mucoproteins have been found either normal (Tyrosine Index <3.5) or moderately high (3.5 to 4.5) or frankly elevated (>4.5). According to the data given in Table I, it may be expected that (a) when the total protein sugar level is normal (*i.e.* between 90 and 120 mg %) only 66.6% of the cases have normal mucoproteins and moreover 20.1% may even have a high concentration; (b) when total protein sugar is more than 160 mg % mucoproteins are abnormal in 98.2% of cases; (c) when total protein sugar is very high (above 180 mg %) mucoproteins are always abnormal and even frankly high in 92.5% of cases.

In 6 patients, total protein sugar and mucoproteins were determined simultaneously before and during the administration of cortisone. The patients were followed from 105 to 250 days, and determinations were performed every 5 to 15 days. The curves obtained, presented in Fig. 1, are grossly parallel but a number of discrepancies may be noted, especially after the patients have been treated for some weeks.

Discussion. In view of their parallelism, it is possible that a common factor regulates mucoprotein and the total protein carbohydrate concentration. Since it is the α-globulin fraction of serum which is richest in carbohydrate, the regulation of mucoprotein concentration may be dependent on a primary regulation of α-globulin synthesis. It may be assumed that this common factor is at work when the anabolic phase predominates since mucoprotein is always high when the total protein sugar is high. Catabolism of mucoproteins may follow a different course from that of the globulins rich in sugar since

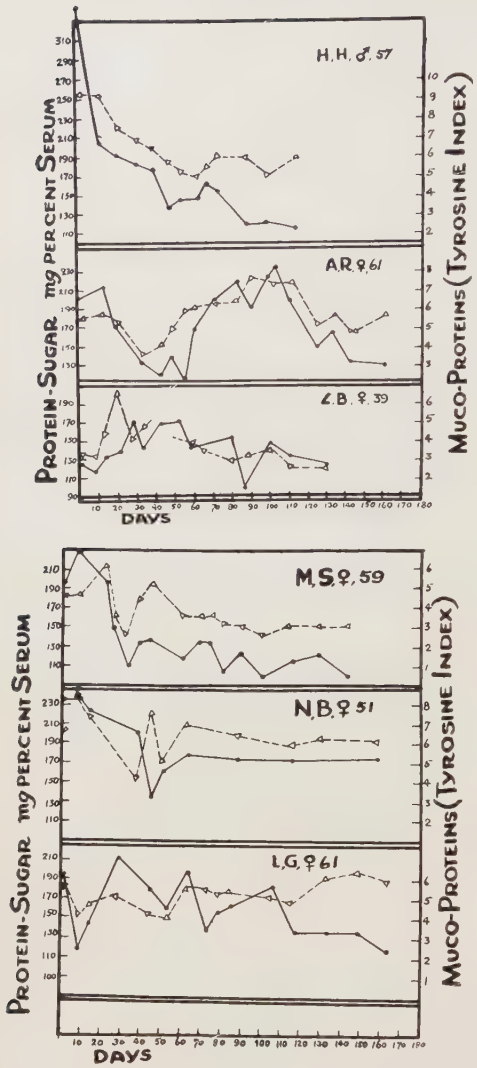

FIG. 1. Variation of mucoproteins and serum protein-sugar levels in 6 patients treated with cortisone.

discrepancies are present mainly when the catabolic phase predominates, *i.e.*, in the falling portions of the curves.

This work confirms the recent findings of Shetlar and his coworkers who have compared different groups of arthritic patients and demonstrated a close parallelism between the average values of mucoprotein sugar and total serum protein carbohydrates(8).

Summary. In 41 patients with rheumatoid arthritis, who were studied before and during the administration of cortisone, serum mucoprotein and the protein-bound carbohydrate concentrations were studied and compared. 1. The correlation coefficient of the 2 tests was found to be moderately high, r = 0.62. 2. It was found that when the total protein sugar concentration was very high, mucoprotein concentration was always above normal values. 3. When the total protein sugar concentration was normal, the serum mucoprotein index was often above normal. 4. From these findings and from the comparative study of the variation of total protein sugar and mucoprotein concentrations during the treatment of 6 patients with cortisone, it is suggested that a common factor regulates the formation of serum mucoproteins and other plasma proteins which are rich in carbohydrate.

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Effect of Partial Shielding by Grids on Survival of X-irradiated Rats. (21036)

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There appear to be at least two general mechanisms by which partial body shielding protects animals from x-radiation. The first of these, which may be termed "remote" effect, has been thoroughly investigated by a number of workers(1-7). In studies on this mechanism of protection a single organ or portion of the body is shielded with lead and the "remote" effects such as effect on survival, white cell count, rate of regeneration of the marrow, etc., are studied. Whether or not the results can be explained by a humoral substance(1), cell seeding(1,6,7), or a temporary maintenance of the animal's defense mechanisms(6) is not yet established. In any event it is clear that reparative processes are stimulated at a distance from, but under the influence of, the shielded normal tissue. The second general mechanism may be called the "local" effect of shielding. Here the area of interest is located at the interface between shielded and irradiated tissues. Kohler(8), Liberson(9), Goldfeder(10), Grynkraut(11), Jolles(12), Marks(13), and others have shown that when radiation is delivered through multiple holes in a shielding material, *i.e.* through a grid, doses which would not ordinarily be tolerated by the skin if given over the entire area can be delivered with relative safety. It is generally believed that this phenomenon can be best explained on the basis of the normal shielded skin(9,14) and/or connective tissue (12) adjacent to the irradiated tissue initiating repair of the damaged area. No information is available as to whether this protective effect is limited to the skin and underlying connective tissue or is a property of tissues in general which might be utilized to protect animals against total body irradiation.

The present study was undertaken to determine whether or not irradiation of entire animals through a grid is less effective in producing lethality than direct total body irradiation and if so, to determine the relation of effectiveness to grid hole size.

Experimental. A total of 104 adult male Sprague-Dawley rats weighing 250 ± 25 g each were used in the present study. Prior to and following irradiation the rats were housed 2 or 3 to a cage and given water and Purina Laboratory chow *ad libitum*. The rats were anesthetized with pentobarbital prior to irradiation exposure. They were then placed, 2 at a time, on their left sides in a shallow lucite cage. A lucite lid, with or without a lead grid attached, was placed on top of the cage in close proximity to the surface of the rats. Following radiation exposure the animals were observed daily for 30 days and the survival noted. The grids used in the present study were constructed from lead sheets 1.6 mm thick. Previous measurements showed that lead of this thickness transmitted only 1% of the incident radiation dose. The closed to open areas were kept constant at 60 to 40%, respectively, and only the hole size was varied. The holes were distributed as uniformly as possible over a circular area of 189 cm^2. X-rays were delivered from a Keleket Deep Therapy Model machine operated at 200 KVP and 9 ma with added filters of 1.0 mm Al and 0.5 mm Cu. The target-specimen distance was 29.5 cm and the dose rate 62.0 r/min. measured in air. The grid transmission dose rates were measured by rotating a Victoreen ionization chamber under the grids on an eccentric axis. Data on hole sizes, transmission rates, etc., for the various grids are given in Table I. To determine the approximate extent of scatter of the radiation in passing through a rat, films were placed immediately beneath the grid and immediately beneath the rats. The assembly was then exposed to radiation. The results of this study indicated that scattering was minimal and that when a grid was used, the radiation was delivered essentially as sharply defined cylinders. Since rats exposed through the grid received only about 40% of the dose incident to the grid, it was necessary to com-

TABLE I. Physical Data on Grid Hole Sizes, % Open Area, and Dose Rates Transmitted.

Shielding	Hole size (diam. cm)	Hole area (cm^2)	Distance between centers (cm)	Calculated % open area	Measured % open area	Dose rate through grid (r/min.)
0 (open portal)				100	100	62.0
Grid 1	1.0	.79	1.40	40	46	28.3
2	.66	.34	.93	40	44	27.5
3	.44	.15	.61	40	42	26.2
4	.32	.08	.44	40	46	28.6

pensate for this lower dose rate to the shielded animals by increasing the exposure times. For this purpose the concept of volume dose as discussed by Hempelmann *et al.*(15) was used. According to these authors the integral or volume dose "is the product of the radiation dose in energy units multiplied by the mass of irradiated tissue in grams." The unshielded control rats (avg wt 250 g) were given a total body exposure of 800 r or an average volume dose of 200 kg roentgens per rat. Approximately 40% or about 100 g of the rats irradiated through the various grids were exposed to radiation. To make the total energy absorption or average volume dose per rat equal to the dose received by the controls it was necessary to increase the dose incident through the holes in the grids to about 1740 to 1900 r (the exact dose depending on the measured percent open area).

Results. Exposure of rats to equal volume doses of x-radiation through grids having various diameter hole sizes but a constant open area to closed area ratio resulted in increasingly greater survival as the hole size was decreased. These data are shown in Table II. Of the 24 control rats exposed to a total body radiation dose of 800 r (or 200 kg roentgens volume dose) only 5 or 21% survived for 30 days. Rats irradiated with the same volume dose (2000 r to 40% of the body or 200 kg roentgens) showed no increase in survival when the radiation was delivered through a grid having holes 1 cm in diameter. When the hole size was reduced to 0.66, 0.44, and 0.32 cm in diameter, however, 30-day survivals of 37, 68, and 84%, respectively, were found. In Fig. 1 the per cent survival is plotted against log of hole areas. The resulting straight line indicates a simple exponential relationship over the range of hole sizes studied.

TABLE II. Effect of X-Irradiation through Various Grids on Survival of Rats. Volume dose 200, kg roentgens/250 g rat.

Shielding	Hole size (diam. cm)	No. rats	30-day survival	% survival
0 (open portal)		24	5	21
Grid 1	1.0	19	3	16
2	.66	19	7	37
3	.44	19	13	68
4	.32	19	16	84

Discussion. Exposure of rats to x-radiation delivered through grids has been shown, in the present study, to increase survival significantly over that found in control rats exposed to equal volume doses of total body x-irradiation. Grid hole size was found to be of critical importance in altering survival. Rats exposed through grids having openings of 0.66 cm or less in diameter showed increasing survival with decreasing hole size even though the ratio of total open area to closed area was kept constant at a 40 to 60% ratio.

Other investigators have found similar protection of skin and underlying connective tissue of men and experimental animals when radiation was delivered through a grid(8,14). Presumably this protective effect, which may be called the local effect of partial shielding as opposed to the remote effects obtained by spleen or marrow shielding, may be explained as being due to the initiation of repair in the irradiated area by the surrounding unirradiated tissues. This conclusion is supported by the fact that greater protection was obtained as hole sizes in the grids were decreased. Such decrease in size had the effect of increasing the total area of the interface between irradiated and normal tissues. If protection is due to reparative changes induced at these interfaces then it would be expected that degree of pro-

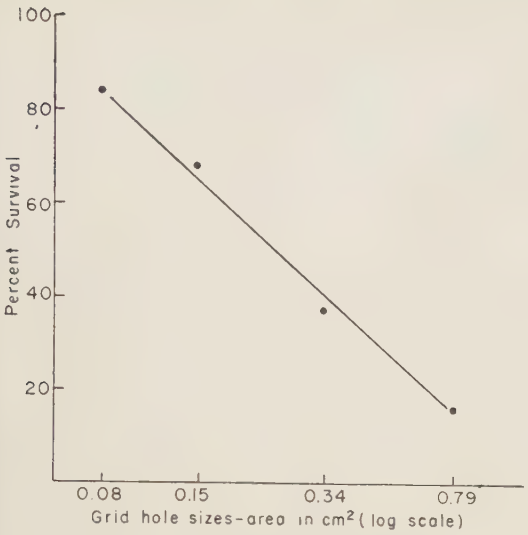

FIG. 1. Survival of rats exposed to equal volume doses of X-rays as a function of grid hole size.

tection would be related to interface area. The data obtained in the present study showed such a relationship (Table II and Fig. 1).

It should be pointed out that this theory may be further tested by changing the shape of the openings in the grids while maintaining the area of the openings constant. Circular holes should give minimum protection since the perimeter to area ratio is smaller than the perimeter to area ratio of other geometrical shapes such as the squares and rectangles that are used by clinical radiotherapists. Further study on this point is indicated.

The possibility that the protective effect obtained in the present study was exerted remotely by protection of hematopoietic and/or lymphatic tissue must be considered. Since the areas of the rat that were shielded were randomly distributed and in each case amounted to 60% of the animal, it is apparent from the laws of probability that approximately 60% of the bone marrow, spleen, lymphatic tissue, etc., were protected. If protection had been obtained by the remote effects of shielding these tissues, then no difference in survival with grid openings of various sizes would be expected. Since differences were found it must be concluded that the protective effect could not be explained on this basis.

Studies in which large single areas of animals have been shielded and the remainder exposed to radiation have shown that in general there is a fairly specific threshold of dosage which cannot be exceeded if the animal is to survive(3). This threshold presumably represents the level at which remote effects of shielding are no longer effective and direct irreversible damage to vital tissues leads to death. The present study suggests that the local effect of shielding may be effective in protecting animals at doses in excess of those at which the remote effects are operative. It seems likely, on the basis of these considerations, that shielding of a given percentage of an animal by a grid may be preferable to shielding a similar percentage of an animal by a single solid sheet of protective material.

Summary. Exposure of rats to equal volume dose of x-radiation (200 kg roentgens) through grids having various diameter hole sizes but a constant open area to closed area ratio resulted in increasingly greater survival as the grid hole sizes were decreased. Significantly greater survival was obtained in rats exposed through grids than in rats exposed to total body x-irradiation. It is suggested that these results may be explained by a beneficial local effect of partial shielding whereby normal tissue adjacent to irradiated tissue initiates repair in the irradiated areas. Degree of survival appeared to be directly related to the area of interface between normal and radiated tissue.

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Normal and Altered Thyroidal Function in Domesticated Goldfish, *Carassius auratus.** (21037)

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The ability of thyroid follicles of fishes to accumulate radioactive iodine appears to be inversely proportional to the amount of iodine in their aqueous environment(1). Thus, it has been shown that marine fishes living in an iodine-rich environment seldom concentrate thyroidally more than 2 to 3% of a tracer dose of I^{131} while freshwater fishes living in a relatively iodine-poor medium accumulate up to 30% of an injected dose of I^{131}. The radioiodine turnover of such a freshwater fish can be reduced until it resembles that of a salt water fish by the addition of iodine to the water(1,8). It also seems to be true that euryhaline fish such as *Fundulus heteroclitus* that live in water which is salty at certain times, and almost fresh at others, can rapidly change their iodine metabolism while strictly marine fish such as *Fundulus majalis* are not so adaptable(5).

In a survey of the radioiodine turnover of freshwater fishes, we have found only one species, the common goldfish *Carassius auratus,* whose thyroid gland is not a relatively avid collector of radioiodine. The thyroidal epithelium of this species is normally squamous, denoting comparative inactivity, but it is extremely sensitive to thyrotropic hormone stimulation, so much so that it has been suggested as the basis for the bioassay of TSH (3,4). We were surprised to find that untreated goldfish make almost no thyroxine within the usual time required for such biosynthesis by other species, and undertook the following series of experiments with thyrotropic hormone to try to alter their exceptionally low level of iodine metabolism.

Material and methods. Eighty-four goldfish were injected intraperitoneally with 5 Junckmann-Schoeller units of thyrotropic hormone (TSH)† per day in .05 cc of 0.9% saline solution. Twenty-four animals received a total of 20 units of thyrotropin in 4 days; 60 others, 25 units in 5 days. The day after the last injection of TSH all were injected with about 20 μc of carrier-free I^{131} and sacrificed in groups of 6 at intervals thereafter up to 170 hours. Ninety-nine fish, used as controls received the tracer dose of I^{131} at the same time as the experimental animals and were sacrificed after a similar interval. Twenty-four additional fish were each injected with 2 frog pituitaries suspended in .05 cc of 0.9% saline solution. Forty-eight hours later they received tracer doses of I^{131}. Another group of 10 fish was kept for 2 months in conditioned aquarium water at the genetics laboratory in the American Museum of Natural History. This water is known to be extremely low in iodine (0.5

* These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Columbia University (NR 163-208)

† The thyrotropic hormone was kindly supplied by Armour and Co. Their preparation number is P 589-80 and is stated to contain 10 J-S units per mg.

μg of iodine/liter as opposed to 2.3 μg/liter in New York City tap water), so low that xiphophorin fishes reared in it routinely develop thyroid tumors(2). Goldfish were kept in the museum aquarium water to see whether the need for more efficient use of available iodine in this extraordinarily iodine-poor environment might stimulate their normally quiescent thyroid glands. The fish were of the "Comet" variety, and were purchased from the Grassyforks Fisheries, Saddle River, N. J. They were kept in all-glass aquaria which were well-aerated by bubbling compressed air. The fish were fed a beef-liver-pablum diet during the period of the experiment(6). At the time of sacrifice all fish were killed rapidly in hot water, their lower jaws containing the thyroid follicles were removed and homogenized in 0.9% saline solution, and kept at 16°C overnight to permit the extraction of the iodinated compounds. Total thyroidal radioiodine content was then determined by measuring radioactivity of aliquots of the saline extract. The pH was adjusted to 8.5, and the homogenates were digested for 24 hours with trypsin and 24 more with papain (at pH 5.3), a modification of the technic of Roche *et al.*(11), and Lissitzky (9). The digested material was extracted first with chloroform to remove fats and then with butanol saturated with N/10 HCl. The acidified butanol, containing the iodinated amino acids, was dried under vacuum and the residue redissolved in 0.1 cc of butanol and analyzed by paper chromatography. N-butanol-acetic-acid and n-butanol-ammonia were used as developing solvents. Monoiodotyrosine (MIT), diiodotyrosine (DIT), triiodothyronine (TITn), thyroxine (Tx) and potassium iodide (KI) were used as known reference standards. For the observation of the distribution of radioactivity along the length of the filter paper chromatograms, a Geiger tube and shielding arrangement resembling that of Lissitzky(9) were used. Radioautographs were made by exposure of paraffin sections of the thyroid tissue of I^{131} injected goldfish to squares of x-ray film.

Results. In all 3 experiments with control goldfish, *i.e.* those not treated with TSH, thyroidal iodine uptake was consistently low

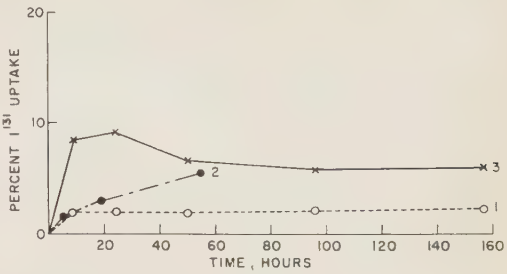

FIG. 1. Effect of thyrotropic hormone on peak accumulation and turnover of I^{131} by thyroid of the goldfish. Curve 1: Untreated goldfish. Peak value, 3% of injected dose of I^{131}, is reached 12 hr after injection. Curve 2: Goldfish pretreated for 48 hr with whole frog pituitaries. Curve 3: Goldfish pretreated 4 or 5 days with mammalian TSH. Note that TSH-treated fishes release about ⅓ of their peak accumulation of radioiodine after 48 hr, while the untreated fish hold 100% of their peak accumulation for the length of the experiment, 156 hr.

(Fig. 1, Curve 1), the maximum uptake was 3% of the injected dose and was reached 12 to 24 hours after injection. The thyroid evidently was not only sluggish in accumulating I^{131} but also was slow in releasing it because 100% of the peak accumulation was still held by the gland at the termination of the experiment 156 hours after it was started. TSH increased the ability of the gland to concentrate radioiodine over 3 times. The greatest uptake seen in any one fish, 17% of the injected dose, was after 5 days of TSH stimulation and 24 hours after I^{131} injection. However, an extra day of TSH treatment made no statistically significant difference in the I^{131} uptake by the thyroid. Therefore, the data on the effects of 4 and 5 days of stimulation by TSH are plotted together as a single curve in Fig. 1, Curve 3. The average uptake reached its peak value, 9% of the injected dose, 24 hours after I^{131} injection. After 156 hours the thyroid still held 66% of its peak accumulation.

Fresh whole frog pituitary preparations were also successful in stimulating the goldfish thyroid (Fig. 1, Curve 2). The response was not as great as with the purified mammalian product, possibly because the frog pituitary hormone was permitted to act for only 2 days.

Finally, the thyroids of the goldfish reared in the aquarium water at the American Mu-

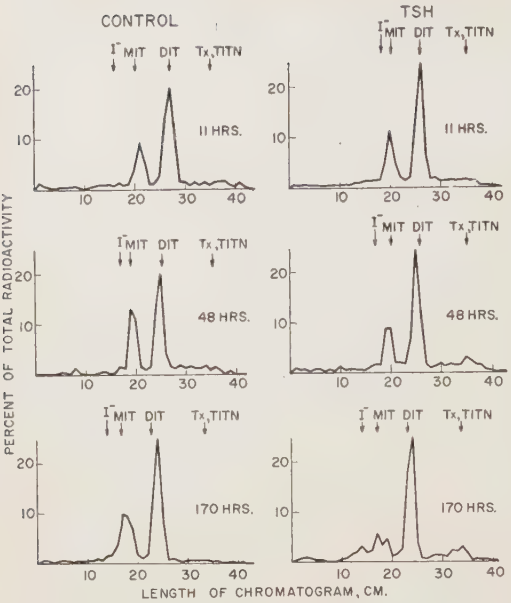

FIG. 2. Radiochromatograms of trypsin and papain hydrolysates of thyroid tissue of control and thyrotropic hormone (TSH) treated goldfish sacrificed at intervals after injection of a tracer dose of radioactive iodine. Figures show distribution of radioactivity along length of chromatograms in which butanol-acetic-acid was the developing solvent. Probable chemical formula of I^{131} is indicated at top of each figure. Arrows indicate position of known iodinated compounds chromatogrammed in parallel with thyroid hydrolysates. I, iodide; MIT, monoiodotyrosine; DIT, diiodotyrosine; TITn, triiodothyronine; Tx, thyroxine. Note absence of thyroxine in control series, and its presence after TSH treatment.

seum of Natural History accumulated 5% of the injected dose of I^{131} in 24 hours. This uptake is 167% of the 24-hour uptake by the fish reared in ordinary tap water.

Radiochromatography. The synthesis of iodinated amino acids followed the same pattern in the TSH-treated goldfish as in many other vertebrates. MIT and iodide predominated in the earlier hours, DIT in the later ones. Thyroxine first appeared in trace amounts between 11 and 24 hours, but was never present in large proportion. Even 170 hours after injection of the isotope it accounted for only 8.3% of the total radioactivity (Fig. 2).

Interestingly enough, in only one of the 3 control runs was there any thyroxine demonstrable. In that one case, 7% of the total radioactivity was radiothyroxine and the amount remained constant from 25 to 96 hours. In the other 2 experiments there was no thyroxine present. Triiodothyronine was absent in all cases.

Radioautography. The follicular epithelium of the control fish was squamous; that of the TSH-treated animals was high columnar. In both cases the thyroidal colloid autographed heavily.

Discussion. Although the function of the piscine thyroid gland is still essentially unknown, it has been assumed that the fish thyroid follicle produces a hormone of some functional importance to the animal(7,10). It is surprising, therefore, that these experiments indicate that for as long as 7 days after it enters the thyroid gland tracer iodine in the goldfish may not be incorporated into either thyroxine or triiodothyronine. It is possible, of course, that the hormone is released into the blood stream immediately upon production and is, therefore, not detectable on chromatograms of thyroid tissue. This, however, seems unlikely, because thyroxine was found in the thyroids of goldfish treated with TSH, an agent which is known to speed the release of thyroxine from the thyroid into the blood stream, at least in mammals.

In addition to being a poor thyroxine-producer the goldfish thyroid is sluggish in its response to iodine levels in the water. It does respond because the I^{131} uptake increases when the fish are placed in water known to be very poor in this element, but the response is not nearly as great as that of other species of fish(1,5). This low efficiency seems to be referable partially to a lack of pituitary thyrotropin because treatment with mammalian or amphibian TSH tends to alter the condition. Furthermore, there is no I^{131} turnover in the untreated animals, while the turnover in the fish pretreated with TSH is typical of that of any other fresh water teleost. Even with TSH stimulation, however, the goldfish thyroid is not as efficient as that known for the average freshwater fish. Possibly teleost TSH would be a more effective stimulant than the mammalian or amphibian material.

The goldfish has long been a domesticated

species living in quiet pools or aquaria. It is possible that the type of selection used by fanciers in developing the present strains of domesticated *Carassius* has in some way been selective also for a given state of thyroidal activity. Possibly it would be illuminating, therefore, to test the thyroidal activity of wild *Carassius,* or of the closely related carp.

Summary. 1. Normal goldfish, *Carassius auratus* accumulate a peak of 3% of an injected tracer dose of I^{131} intrathyroidally. This small amount of radioiodine remains in the thyroid at the same level for more than a week. Thyroids of thyrotropic hormone-injected goldfish accumulate 9% or more of the tracer I^{131}, but lose at least 40% of it within a week, indicating a greater turnover rate. 2. Radioiodine in normal goldfish thyroids is mostly in the form of monoiodotyrosine and diiodotyrosine, with little, if any, thyroxine formed even after one week. The thyrotropic hormone stimulated goldfish thyroid forms a small proportion of radiothyroxine as early at 24 hours after injected I^{131} and as much as 8% of the I^{131} is in the form of thyroxine by 7 days after injection of I^{131}. 3. Keeping the goldfish in water low in iodine had an effect on I^{131} metabolism similar to thyrotropic hormone treatment.

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Protective Effect of Hypoxia against Irradiation Injury of the Rat Bone Marrow and Spleen.* (21038)

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The reduction in radiation sensitivity by hypoxia has been demonstrated under a variety of experimental conditions(1). Dowdy, Bennett, and Chastain(2) have shown that the LD_{50} dose of x-irradiation is increased from 800 r to between 1200 and 1400 r when rats are irradiated in a 5% oxygen atmosphere.

In previous studies reported from this laboratory(3-5), the concentration of desoxyribonucleic acid has been used as a measure of the cellularity of the bone marrow and spleen and the rate of incorporation of radiophosphorus into this fraction as an index of mitosis rate. In the present investigation, these methods have been used in a study of the protective effect of hypoxia against irradiation damage to these organs.

Methods. Male rats of the Sprague-Dawley strain, 3 to 4 months old with an average weight of 290 g were used. Hypoxia was produced by submitting the animals to a stimulated altitude of 25,000 feet, previously described(5). Irradiated animals were given 800 r of total body irradiation at the rate of 21 r per minute from a 220 kv Maximar

* This study supported by funds under contracts between the USAF School of Aviation Medicine, Randolph Field, Texas, and between the U. S. Atomic Energy Commission and Northwestern University, and by the Armour Laboratories.

† Markle Scholar in Medical Sciences.

TABLE I. Effect of Hypoxia on DNA Metabolism in Rat Bone Marrow and Spleen following Irradiation.

Group	No.	Phosphorus content, mg/g ASP	Phosphorus content, mg/g DNAP	Specific activity ASP	Specific activity DNAP
		Bone marrow			
Normal	15	.966 ± .088	1.320 ± .295	.481 ± .064	.199 ± .034
Irradiation alone	5	.676 ± .218	.109 ± .088	.366 ± .070	.060 ± .034
" + hypoxia	7	.699 ± .088	*.208* ± .069	.527 ± .062	*.190* ± .057
Irradiation + hypoxia following intermittent hypoxia	6	.525 ± .135	.082 ± .022	.530 ± .141	*.167* ± .049
		Spleen			
Normal	15	.969 ± .107	1.294 ± .244	.468 ± .011	.034 ± .002
Irradiation alone	5	.926 ± .036	.281 ± .037	.635 ± .083	.003 ± .003
" + hypoxia	7	.921 ± .063	*.475* ± .067	.637 ± .061	*.037* ± .014
Irradiation + hypoxia following intermittent hypoxia	6	.840 ± .042	*.460* ± .112	.613 ± .049	*.035* ± .022

Italics indicate significant differences from control irradiated animals (P value >0.98).

operating at 20 M.A. Filtration used was 0.5 mm of copper and 1.0 mm of aluminum. The HVL was 1.15 mm of copper and the target distance was 70 cm. The animals were divided into 4 groups: the first group was untreated and served as a control; the second was irradiated at normal atmospheric pressure; the third group was subjected to hypoxia only while being irradiated; the fourth group was submitted to 10 hours of hypoxia daily for 3 days and 15 hours after completion of the period of intermittent hypoxia were irradiated in a hypoxic atmosphere. Following irradiation all animals were returned to their cages and 96 hours later were injected intraperitoneally with 2 μc of carrier-free $NaH_2P^{32}O_4$‡ per 100 g of body weight. Four hours after administration of the isotope the animals were sacrificed by a blow on the head and tissues removed for chemical, radioisotopic, and morphologic studies by methods previously described(5). The values for acid soluble phosphorus (ASP) and DNAP content are expressed as mg of phosphorus per g wet weight of tissue. The specific activity represents the per cent of the injected dose of radiophosphorus present/mg of phosphorus in the fraction. The significance of differences between the hypoxic animals and control irradiated animals were subjected to "t" analysis by the method of Fisher(6). Differences were considered significant if the probability was 0.98 or greater.

Results. Ninety-six hours post-irradiation the DNAP concentration of the bone marrow and spleen was markedly depressed but was significantly higher in the animals irradiated in a hypoxic atmosphere than in the controls (Table I). The cellularity as determined by direct counting on imprints followed closely the changes in the DNAP concentration. The rate of incorporation of the P^{32} into the DNA (specific activity) of the bone marrow and spleen was near normal in the "hypoxic" animals but was markedly depressed in the irradiated controls.

Preliminary exposure of animals to intermittent hypoxia before irradiation at reduced oxygen tension does not result in any additional protection to the bone marrow and spleen over that observed in animals subjected to hypoxia only during irradiation (Table I). The differential counts of cells in the bone marrow and spleen in normal, control irradiated and protected animals is shown in Table II.

Discussion. The protective effect of hypoxia has been attributed to the reduction in the number of free radicals produced from the interaction between the radiation and water in the biological system(7). In theory hypoxia would reduce the biological effect of a given dose of x-irradiation to that which would be produced by a lower x-ray dose without hypoxia. The pattern of DNAP con-

‡ Obtained on allocation from the U. S. Atomic Energy Commission.

TABLE II. Effect of Hypoxia on Differential Counts in Rat Bone Marrow and Spleen following X-Irradiation.
Major cell types in %.

Group	Bone marrow				Spleen			
	E	G	L	R.E.	E	G	L	R.E.
Normal	46	32	16	.3	5	8	82	4
Control x-ray	1	20	2	59	0	4	5	91
Acute hypoxia	1	56	2	15	1	6	30	61
Chronic "	7	43	2	34	0	5	8	86

E = Erythrocytic series; G = Granulocytic series; L = Lymphocytic series; R.E. = Reticuloendothelial cells.

centration, specific activity and distribution of cell types in the bone marrow and spleen in the animals receiving 800 r at low pressures corresponds to that which would be expected in unprotected animals receiving 600 r(8). Thus hypoxia appears to have reduced the biological effectiveness by 200 r as measured by its action on the bone marrow.

Previous investigators have found a high radiosensitivity of cells of the erythrocytic and radioresistance of cells of the reticuloendothelial series(9). The findings are substantiated in the present study and there was no significant difference between the control irradiated animals and the protected animals. This indicates that hypoxia does not alter the relative radiosensitivity of the various cell types present in the bone marrow and spleen. Although there is little difference in the degree of hypocellularity that develops in the control irradiated and protected animals, the return of mitosis in the latter group probably represents one manifestation of the protective action of hypoxia.

We have observed previously that 30 hours of intermittent hypoxia produced a moderate increase in cell mitosis in the bone marrow and a marked increase in mitosis in the spleen (5). The failure of hypoxia induced stimulation of the bone marrow and spleen to increase protection following irradiation was probably due to the relative sensitivity of the young dividing cells of the erythrocytic series.

The results obtained in animals exposed to hypoxia only during irradiation suggests that the protection observed by Jacobson *et al.* (10) in animals pretreated with phenylhydrazine may have been due to tissue hypoxia resulting from the anemia present in these animals rather than to the hyperplastic bone marrow as they suggested.

Summary. 1. Rats irradiated in a hypoxic atmosphere show a slightly higher cellularity and much greater rate of DNA synthesis in the bone marrow and spleen after 96 hours than rats irradiated at ground level. 2. Thirty hours of intermittent hypoxia preceding irradiation at low oxygen tensions does not give additional protection. 3. A striking parallelism exists in the cellular reactions in the bone marrow and spleen following irradiation, both at normal atmospheric pressures and at low oxygen pressure. 4. Irradiation of animals at low oxygen pressures appears to reduce the biological effectiveness of 800 r on the bone marrow to 600 r.

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Effects of Pantothenic Acid and Its Analogs in Radiation Injury by P^{32}.* (21039)

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In the first paper of this series it has been shown that the severity of the damage caused by the administration of P^{32} can be modified by changes in the levels of dietary protein, fat, or phosphate(1). Subsequently, we investigated the role of certain B vitamins, a deficiency of which is less likely to occur under ordinary nutritional conditions. Beneficial effects were observed with the administration of vit. B_{12} and folic acid(2), or of pyridoxin(3), to mice deficient in these vitamins. The results of the present investigation, which concludes this phase of our study, indicate that pantothenic acid (PA) also exerts a significant protection against the injurious effects of the radioisotope. The sulfonic acid analog of PA, pantoyltaurine (PT), was found to be almost as active in this respect as the natural vitamin, whereas no protection was apparent with ω-methyl pantothenate (MP), an analog in which the pantoyl moiety of PA has been modified.

Experimental. The basal diet (Diet 45) contained: Casein 12 p.p. 100, dextrin 26, sucrose 25, Crisco 25, cod liver oil 5, Ruffex 2, Salt Mixture (U.S.P. No. 2) 4, sulfasuxidine 1, or 1.5. To each 100 g portion of the diet, B vitamins were added in the following amounts: Thiamine HCl 0.4 mg, riboflavin 0.8 mg, pyridoxine HCl 0.4, niacin 5.0, folic acid 0.1, menadione 0.5, biotin 0.05, B_{12} 0.02, inositol 10. Other supplements which were added to the diet of some of the animals only, were: Ca pantothenate (PA) 5 mg p. 100 g, p-aminobenzoic acid (PABA) 2.5 mg, pantoyltaurine (PT) 100 mg, taurine (T) 30-100 mg, ω-methyl-pantothenate (MP) 25 mg. Male albino mice, with an initial average weight of 20 g, were distributed in groups of 8 or 9, and each group housed in a metallic cage with raised screen floor. The diet, to which vitamins and supplements were freshly added, was given 3 times a week, and food consumption was determined. The number of mice alive in each day of experiment and the weekly changes in body weight were also recorded. Seventeen series of experiments were carried out on a total of about 2000 animals, each series including several groups under different nutritional conditions. After a period varying between 5 and 10 days on the experimental diet, some groups were injected intraperitoneally with a solution of phosphate (pH 7.4), containing 0.5 millicuries/ml. The doses injected in different series varied between 4.5 and 5.5 μc/g of animal. Control groups were injected with an identical volume of Ringer solution. Since, in preliminary experiments, the control animals on the PA-deficient diet for more than 4 weeks exhibited a high mortality, in the subsequent series PA was added to the diet of all groups, beginning with the 21st day after the injection of P^{32}. Administration of supplements other than PA was also discontinued at the same date. Under these conditions, very few deaths, or no deaths at all, occurred among the controls, not injected with P^{32}. The mice were kept on the diet and observed for 6 weeks after the injections.

Results. As in previous papers(1-3), the time of 50% deaths, the % of survivors at the 21st day, and the average length of survival were taken as indications of the damage caused by the isotope, a 42-day survival being ascribed to the mice which were still alive at the end of the experiment. All data have been corrected for the mortality of the control groups, not injected with P^{32}.

In Tables I-IV, the average values obtained in several series of experiments from the

* Technical assistance was given by C. Downs. This work was performed under a contract between the U. S. Atomic Energy Commission and the Bowman Gray School of Medicine. The P^{32} was obtained from the Oak Ridge National Laboratories under allocation of the U. S. Atomic Energy Commission.

TABLE I. Effects of Pantothenic Acid (PA) on Survival of Mice Injected with P^{32}.

Series	Dietary supplement PA	No. of mice	Time of 50% deaths, days	Survivors 21st day, %	Avg length of survival, days*
1- 7†	—‖	172	16	32	23 ± .8
	+	167	>42	69	32 ± 1.0
8-13‡	—‖	163	20	49	26 ± 1.0
	+	153	>42	62	30 ± 1.1
14-15§	—‖	39	17	41	22 ± 1.9
	+	40	29	65	30 ± 2.0

* Values preceded by ± are stand. errors of the means.

† Diet 45 + sulfasuxidine 1.5%. PABA included in vit. sol.

‡ *Idem.* PABA excluded from vit. sol.

§ Diet 45, without sulfasuxidine. PABA included in vit. sol.

‖ PA was added beginning 21st day after inj. of P^{32}.

groups receiving, or not receiving, a given supplement, have been compared. For such a comparison only the data obtained from groups, run simultaneously and injected with the same batch of P^{32}, were used. This should minimize the effects of slight changes in the conditions of the various experimental series, and also of possible variations in the actual potency and chemical purity of the isotopic solutions injected. From Table I it is apparent that administration of PA increased the survival of mice on the basal diet, supplemented with sulfasuxidine. The differences between the groups receiving, or not receiving, PA were somewhat smaller in series 14-15 in which sulfasuxidine had been omitted from the diet, and even more so in series 8-13, in which PABA had not been included in the vitamin mixture. The latter finding was further investigated in experiments, in which groups of mice receiving, or not receiving, either or both vitamins were run simultaneously. However, from the results of these directly comparable experiments (which are omitted for brevity sake), it does not appear that the protective action of PA is modified appreciably by the presence or absence of PABA in the vitamin mixture. Table II shows that PT, added to the PA-deficient diet, exerts a protection which is approximately of the same degree as that exerted by the natural vitamin. The values for the survival of mice receiving both PA and PT were about the same as, or higher than, the corresponding figures for the groups receiving PA only. The possibility that the protective action exhibited by PT might be due, at least partly, to the S-containing moiety of PT was tested in experiments in which free T was added to the diet. The data of Table III suggest that the compound might be effective, although at a slight degree only. On the other hand, no protection was apparent with the administration of MP, another analog of PA in which the pantoyl moiety has been modified (Table IV). In the amounts we used, the compound did not seem to be toxic, since no deaths occurred in the control groups, receiving MP, with or without PA. The extremely low acute toxicity of MP has been noted by others(12).

Many of our data have been appraised statistically, using the χ^2 method for the number of survivors at the 21st day, and the t test of significance for the average survival time. The most pertinent results of this appraisal are recorded in Table V.

Discussion. The results of the present

TABLE II. Effect of Pantoyltaurine (PT) on Survival of Mice Injected with P^{32}.

Series*	Dietary supplements PA	PT	No. of mice	Time of 50% deaths, days	Survivors 21st day, %	Avg length of survival, days†
1, 3, 5, 8, 11, 12	—‡	—	136	17	34	22 ±10
	—‡	+§	123	41	61	31 ± 1.1
	+	—	131	>42	57	30 ± 1.1
1, 3, 8, 12	+	—	96	19	47	28 ± 1.4
	+	+§	83	27	55	27 ± 1.8

* Diet 45 with sulfasuxidine 1.5%.

† Values preceded by ± are stand. errors of means.

‡ PA added beginning 21st day after inj. of P^{32}.

§ PT discontinued after 21st day from inj. of P^{32}.

TABLE III. Effects of Taurine (T) on Survival of Mice Injected with P^{32}.

Series*	Dietary supplements PA	T	No. of mice	Time of 50% deaths, days	Survivors 21st day, %	Avg length of survival, days†
4, 5, 11, 13	—‡	—	106	16	39	22 ± 1.2
	—‡	+§	91	20	47	26 ± 1.3
	+	—	101	>42	55	29 ± 1.5

* Diet 45 with sulfasuxidine 1.5%.
† Values preceded by ± are stand. errors of means.
‡ PA added beginning 21st day after inj. of P^{32}.
§ Admin. of T discontinued after 21st day from inj. of P^{32}.

study indicate that the injurious effects of internal radiation by P^{32} are more severe in mice on a PA deficient diet and that administration of this vitamin protects the animals to a significant degree. The finding of a protection by PT was unexpected, since the latter compound is an efficient PA displacer for several microorganisms(4-6). A deficiency syndrome has been described in mice receiving PT(7), but this finding was not duplicated by other workers in mice and hamsters(8), or in rats(9). As far as protection against internal radiation is concerned, PT, in the doses used by us, not only did not behave as an antagonist of PA, but seemed to be able to substitute for the latter quite efficiently. It is possible, however, that the effect of PT is partly due to the taurine moiety, since free T seemed to give some protection. The other analog of PA which we have tested, MP, is quite active in preventing growth of microorganisms which require preformed PA(10) and can also induce a PA deficiency in mice(11). Unlike PT, in our experiments MP did not give any protection against, or perhaps made more severe, the effects of P^{32} in PA deficient mice.

Since PA is a component of Coenzyme A (CoA), it may be postulated that its protective action is due chiefly to the maintenance of an adequate level of CoA in tissues. In this respect, our results can be compared with the finding that, on a molar basis, CoA exerts a greater protection against X irradiation than β-mercaptoethylamine(13).

The results of our present and previous papers indicate that PA, pyridoxin, or the association of folic acid and B_{12} increase significantly the survival of vitamin-deficient mice, injected with P^{32}. A similar effect could not be demonstrated with the administration of other dietary factors, such as choline(1), vit. K and biotin(2), or PABA (this paper). Likewise, in unpublished experiments from this laboratory, α-tocopherol did not alleviate, and, at higher doses, even increased the severity of the damage by the radioisotope. These results closely resemble those obtained by others(14) after whole body X irradiation of mice. It seems, therefore, that certain vitamins play a greater, or a more specific, role in protecting against, or in favoring the recovery from, the injurious effects of radiation.

Our findings may also have some practical significance for the treatment of radiation

TABLE IV. Effect of ω-Methylpantothenate (MP) on Survival of Mice Injected with P^{32}.

Series*	Dietary supplements PA	MP	No. of mice	Time of 50% deaths, days	Survivors 21st day, %	Avg length of survival, days†
15-17	—‡	—	35	30	60	26 ± 1.3
	—‡	+‖	54	24	52	23 ± 1.6
	+	—	26	39	78	33 ± 2.0
	+§	+‖	31	33	62	28 ± 2.5

* Diet 45 with sulfasuxidine 1%.
† Values preceded by ± are stand. errors of means.
‡ PA added beginning 21st day after inj. of P^{32}.
§ These groups received 10 mg/100 g diet, reduced to 5 mg after 21st day from inj. of P^{32}.
‖ MP discontinued after 21st day from inj. of P^{32}.

TABLE V. Statistical Appraisal of Protection by Pantothenic Acid (PA), Pantoyltaurine (PT) or Taurine (T) against Toxic Effects of P^{32}.

Dietary supplement	No. of mice	Survivors at 21st day Chi²	P*	Avg length of survival R†	P*
PA‡	339	61.4	<.001	7.1	<.001
PA§	316	5.5	<.02	2.9	<.01
PA‖	79	4.6	<.05	3.0	<.01
PT	259	19.1	<.001	5.9	<.001
T	197	1.4	>.05	2.4	<.02

* Probability for a chance occurrence.
† Ratio of difference between adjusted means to its stand. error.
‡ PABA included in vit. mixture.
§ " excluded from vit. " .
‖ " included in vit. " . Sulfasuxidine not added to diet.

injury. It seems unlikely that a deficiency of PA, pyridoxine, B_{12}, or folic acid will develop in humans under ordinary conditions, in view of the wide occurrence of these vitamins in natural foods, as well as of their extensive synthesis by the bacterial flora, perhaps by the tissues also. It is possible, however, that, in the radiation syndrome, diminished food intake, impaired absorption from the intestine, severe damage to other tissues, and, in addition, administration of large amounts of antibiotics might lead to an actual deficiency. In such a condition there would be a definite indication for the administration of generous amounts of those vitamins which, in our experiments, appeared to exert a beneficial action.

Summary. A significant protection against the injurious effects of P^{32} was observed by the administration of pantothenic acid to mice on a deficient diet. Pantoyltaurine was almost as effective in this respect as the natural vitamin. ω-methyl pantothenate was totally ineffective.

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Performance of Acclimatized Mice at Altitude. (21040)

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One of the most impressive experiences that one has who journeys to high altitudes and remains there for awhile is the increasing subjective sensation of well being from that of initial feeling of distress. Each day physical work becomes a little easier and mental attitudes become a little less confused. It is well established even in the transient sojourner at natural high altitudes that acclimatization is an important factor in his ability to perform well(1). There are, however, some questions which are not completely answered. How much does acclimatization at a lower altitude increase performance at a

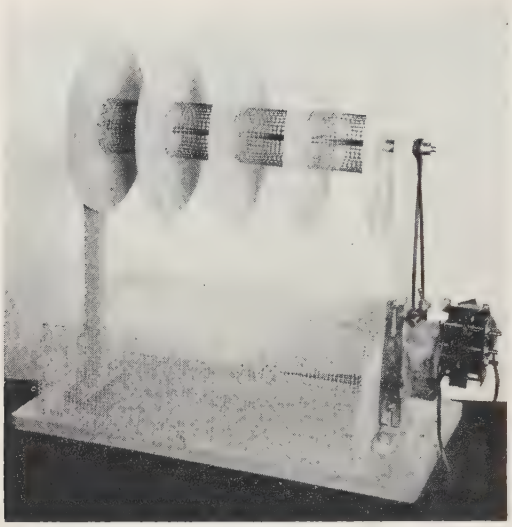

FIG. 1. Photograph of treadmill used for performance tests. Each compartment was 6 inches in width.

higher altitude? How important is the increase in erythrocytes and hemoglobin produced at one altitude to the performance of the organism at a higher altitude? Does the organism lose the advantages gained slowly or rapidly? To test ideas along these lines an experiment was carried out on a group of white mice which were acclimatized in a low pressure chamber.

Procedure. Fifty white mice from the Duke University colony of the New York State Hygiene Dept. strain were divided into 2 groups. One group was kept as ground level or as unacclimatized controls and the other group was slowly acclimatized to a simulated altitude of 20,000 feet (350 mm Hg). All mice were fed the same type of food, given adequate water, and kept at the same environmental temperature. Acclimatization was accomplished in the following manner: The first day the test mice were kept in the low pressure chamber at a pressure of 550 mm Hg and the pressure was reduced by about 30 mm Hg each succeeding day until the pressure of 350 mm Hg was reached on the eighth day and the pressure kept constant at that point for the rest of the experiment. Each day the pressure was lowered to ground level, cages were cleaned and fresh food and water placed in the cages, and the animals weighed. This took approximately one hour. During this period the mice were taken into a large low pressure chamber to 28000 feet (247 mm Hg) and subjected to performance tests. During ascent and descent to the test altitude mice were kept in cages in which a gas mixture of air and oxygen prevented them from becoming hypoxic. The mice were subjected to ambient air during the tests and were in an hypoxic environment only during actual performance tests. Control and acclimatized mice were treated exactly the same. The performance tests were made as follows: One mouse was placed onto one compartment of a motor driven treadmill and kept there until it fell off. This treadmill, which is shown in Fig. 1, had 4 compartments. The treadmill was driven at a constant speed of 12 R.P.M. The tracks on which the mice moved were constructed of hardware screen cloth, 3/16 inch mesh. Mice have an inherent fear of falling and will run or cling to this screen until exhausted. When they were exhausted they fell onto a platform below the mill and were then placed in a jar containing oxygen where they were resuscitated. The exact time of performance on the treadmill was determined by stopwatch by the observer. To eliminate any training error the control mice were tested each time the acclimatized mice were tested. All groups were exercised on the treadmill at ground

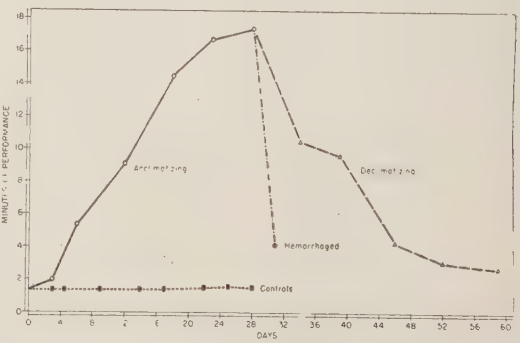

FIG. 2. Graphical representation of results of altitude performance tests on experimental mice subjected to a pressure of 247 mm Hg. At the end of the thirtieth day acclimatization was discontinued and 12 of the acclimatized mice were bled and the remainder (13) were returned to ground level. Test continued as indicated. Points plotted are average values for each group.

TABLE I. Total Hemoglobin Concentration in Blood of Ground Level Control and Altitude Acclimatized Mice.

	g Hb/100 ml blood
Control mice	13.8 ± 1.7 (S.D.)
Acclimatized mice	
1st bleeding	21.1 ± 2.3 (S.D.)
2nd bleeding	11.1 ± 3.4 (S.D.)

level for 10 minutes each day to further reduce a difference in training. There were no significant differences in weights of controls and acclimatized animals during the experiment. When tests had proceeded for 28 days, one group of the acclimatized mice were bled from a tail blood vessel and the hemoglobin concentration determined by a met-cyan hemoglobin method(2) using an Evelyn photoelectric colorimeter. The controls were likewise bled and hemoglobin determinations made. The remainder of the mice which had been acclimatized were then returned to ground level. Performance tests were continued on both controls and the previously acclimatized group over a period of 2 weeks. The mice which had been bled were also tested again over a 4-day period and then re-bled.

Results. The results of these tests are shown in Fig. 2 and Table I. It will be seen that the acclimatized mice showed a rapid increase in performance when subjected to an ambient pressure of 247 mm Hg as compared with unacclimatized mice. Every mouse showed an increase in performance but some more than others. The control group showed no significant change in performance indicating that training was not a factor in these tests.

These results also show that the reverse of acclimatization proceeds rapidly. They also show that removal of blood lowers the level of performance. Perhaps the more interesting aspect is that the mice which were bled did not return to the same level of performance as the unacclimatized controls, but showed a higher level of performance. Analyses showed that their hemoglobin concentration after bleeding was lower than the controls even though their performance was much better. This would seem to indicate that that hemoglobin increase during acclimatization is not the sole factor in accounting for increase in performance at altitude.

Summary. Mice acclimatized to one altitude (350 mm Hg) showed an increase in performance when exposed to a higher altitude (247 mm Hg) as compared with ground level controls. When acclimatized mice are bled they showed a decrease in their performance level. This decrease in performance is not in proportion to the blood withdrawn indicating that an increase in hemoglobin concentration is probably not the sole criterion for an increase in performance at altitude.

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Hemolytic Activity of Diethylstilbestrol and Some Steroid Hormones.* (21041)

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During the course of studies of effects of steroid hormones on influenza virus increase in tissue culture, it was found that when chorioallantoic membranes of 11-day-old chick embryos were incubated with 3.88 x 10^{-4} Mol of diethylstilbestrol, progesterone, testosterone, α-estradiol, DOC, or DOCA, chick chorioallantoic membranes became completely blanched after 24-36 hours of incubation at 35°C and supernatant fluids contained significantly more hemoglobin than control media(1). This phenomenon was observed repeatedly, and suggested that the hormones cited might have hemolytic activity at these low concentrations. Experiments confirming the hemolytic effect of certain of these hormones are reported here.

Materials and methods. Red blood cell suspensions. Human type "O" erythrocytes were used throughout these experiments. Blood was obtained in syringes previously rinsed with ACD (acid citrate dextrose soluton). Whole blood was stored at 4°C in an excess of ACD prior to use—never longer than 48 hours. On the day of experiment cells were washed 3 times with 0.85% NaCl solution buffered to pH 7.14 with 0.01 M phosphate, then suspended in this solution in 10% concentration. RBC concentrations were checked by colorimetric estimation of the hemoglobin concentration of hemolyzed cells. *Hormones* were dissolved in 75% ethanol; addition of hormone solutions to test systems resulted in 10^{-2} dilution yielding final ethanol concentrations of 0.75%. If control tubes containing this concentration of ethanol showed more than 3% hemolysis after 2 hours at 37°C, experiments were discarded. *Determination of degree of hemolysis.* After appropriate incubation, tubes containing hemolytic systems were immediately centrifuged to remove unhemolyzed cells at 1500 RPM for 10 minutes at 4°C. One ml of supernatant fluid was then added to 4 ml of 0.6% NH_4OH solution and the quantity of oxyhemoglobin determined in the Coleman Jr. spectrophotometer at 540 mμ or in the Klett-Summerson Photometer using a No. 54 green filter. Control tubes for assessment of 100% hemolysis contained cell suspensions hemolyzed with distilled H_2O. *Procedure.* To tubes containing 1.8 ml of phosphate buffered saline, 0.2 ml of 10% red cell suspensions were added. Test compounds were added in 0.02 ml amounts with a micropipette.

TABLE I. Comparative Hemolytic Activities of Stilbestrol and Various Steroids.

Compound (3.88×10^{-4}M)	% hemolysis —Hr of incubation— 3	96*	120*
Ethanol control	<1	<1	1
Stilbestrol	100	—	—
Progesterone	92	—	—
DOCA	16	100	—
Testosterone	2	—	6.5
α estradiol	2	—	3.1
Cortisone	<1	—	3.0
Compounds A, B, F	<1	—	—

* Under sterile conditions with penicillin and streptomycin.

Results. Hormones inducing hemolysis. Paralleling evidences of cell toxicity manifest in tissue culture, stilbestrol and the steroids progesterone and DOCA induced hemolysis within a 3-hour period at 37°C (Table I). The sex hormones testosterone and α-estradiol induced only slight hemolysis after prolonged incubation. In the concentrations used (3.88 x 10^{-4} M), none of the corticosteroid hormones manifested hemolytic activity. However, hemolysis by stilbestrol was accelerated by the addition of corticosteroids to the hemolytic system; suggesting a possible hypolytic effect of the latter. Hypolytic concen-

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

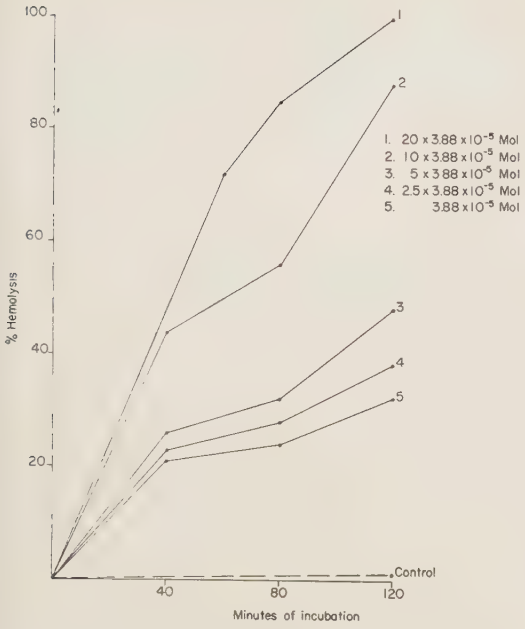

FIG. 1. Relation of stilbestrol concentration to rate of hemolysis.

trations of stilbestrol, and equivalent amounts of cortisone had no effect on the osmotic fragility of erythrocytes.

Hemolysis by stilbestrol. The hemolytic activity of stilbestrol was studied in some detail. Hemolysis was found to bear a direct but non-stoichiometric relationship to the concentration of drug employed (Fig. 1). It should be remarked that complete solution of stilbestrol was not effected at the 2 highest concentrations.

The effect of *temperature* on stilbestrol-induced hemolysis is demonstrated in Fig. 2. Maximum hemolysis was noted at 37.5°C; higher temperatures induced hemolysis in control tubes. Hemolysis was increased by hydrogen ion concentrations below neutrality, as shown in Table II, in which the influence of pH is detailed. Conversely, the addition of Ca in a final concentration of 11 mg % was found to retard the hemolysis induced by

TABLE II. Effect of pH on Stilbestrol-Induced Hemolysis.

	pH				
	5.5	6.0	6.6	7.1	8.0
% hemolysis (40′ at 37.5°C)	98	98	32	28	26

stilbestrol. The inhibitory effect of serum on several types of hemolysis is well known(2). Studies of the *effect of serum* revealed inhibition of stilbestrol hemolysis by final serum concentrations in excess of 5%. Human serum was heated at 56°C for 30 minutes for these experiments. *Prolytic potassium loss.* Prior to the appearance of detectable hemolysis, increased concentrations of potassium were detected in cell suspensions to which stilbestrol had been added. Reactions were carried out at 4°C for 20 hours. In a representative experiment, the addition of 9.7 x 10^{-5} M of stilbestrol resulted in a concentration in the suspending medium of 0.17 meq/L

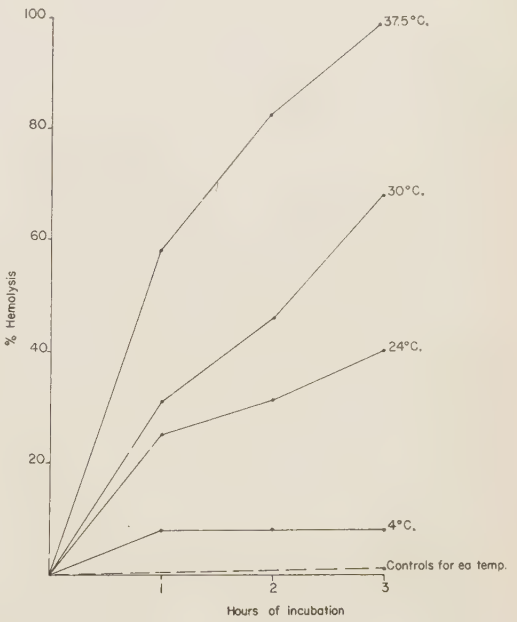

FIG. 2. Effect of temperature on hemolysis by stilbestrol (3.88×10^{-4}Mol).

of potassium compared with 0.04 meq/L in control tubes.

Red cell volume changes. During hemolysis microscopic examination of non-hemolyzed cells disclosed an increase in the size of cells in stilbestrol-treated tubes.

Summary. Diethylstilbestrol, progesterone and DOCA induced *in vitro* hemolysis of human "O" erythrocytes. The hemolytic activity of stilbestrol was modified by temperature, pH, human serum, or the addition of non-hemolytic corticosteroids. Prolytic loss

of potassium from stilbestrol-treated cells was demonstrated.

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Effects of Polyoxyethylene Sorbitan Monolaurate (Tween 20) upon Gastrointestinal Iron Absorption in Hamsters.* (21042)

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In this study iron[59] has been utilized to evaluate the effects of feeding polyoxyethylene sorbitan monolaurate (Tween 20) upon iron absorption. The experiments were performed because Eagle(1) observed that prolonged feeding of rations containing 5, 10 or 15% Tween 20 or other polyoxyethylene derivatives to golden hamsters resulted in consistent hemosiderosis of the cecum, liver and mesenteric lymph nodes and frequently in pigmentary cirrhosis.

This finding may be of some practical importance since polyoxyethylene and sorbitan compounds with fatty acids have been proposed as emulsifiers in many human foods. The theoretical implications are also evident, since investigation of the mechanism of development of these lesions may be helpful in understanding the pathogenesis of hemochromatosis and may increase knowledge of the factors regulating iron absorption from the gastrointestinal tract.

Methods and materials. Three experiments with similar plan have been performed. In each experiment adult hamsters of similar age and weight were divided into 2 groups. One group received a bread ration containing 5% Tween 20 while the second group was given a similar control ration without the Tween. In the first experiment the Tween 20 replaced some of the fat in the experimental ration while in the latter 2 experiments it was incorporated in the diet in addition to the lipid. The compositions of the rations, which were modeled after those described by Eagle are shown in Table I. The iron content of the salt mixture was such that each animal received at least 0.004 g of dietary iron (as ferric salts) per day. To prepare the rations the dry ingredients were mixed and the fat and/or Tween 20 was blended with them. The yeast was dispersed in the required amount of water and this was mixed with the other ingredients to make a dough. This was

TABLE I. Diet Compositions.

Ingredients	Tween ration Exp. 1 (g)	Tween ration Exp. 2 & 3 (g)	Control ration (g)
Vegetable fat	0	5	5
Tween 20	5	5	0
White flour	75.7	75.7	75.7
Baker's yeast	1.5	1.5	1.5
Salt mixture*	1	1	1
Lactalbumin	9	9	9
Sucrose	2	2	2
Water to make dough	56.6 ml	56.6 ml	56.6 ml
Added to ground bread			
Salt mixture*	3	3	3
Wheat germ oil	1	1	1
Vit. A & D oil	1	1	1
Vit. mixture†	0.8	0.8	0.8
Total	100	100	100

* Jones and Foster(2).

† Thiamin HCl 80 mg, riboflavin 80 mg, pyridoxine HCl 80 mg, calcium pantothenate 440 mg, niacin 400 mg, biotin 1.0 mg, folic acid 2.5 mg, 2-methyl 4-naphthoquinone 100 mg, choline chloride 10 g, para-aminobenzoic acid 1.0 g, inositol 1.5 g, Wilson 20:1, liver powder 70 g.

* This investigation was supported in part by Research Grants from the National Institutes of Health, Public Health Service.

† Work done during the tenure of a Damon Runyon Senior Clinical Research Fellowship of the American Cancer Society.

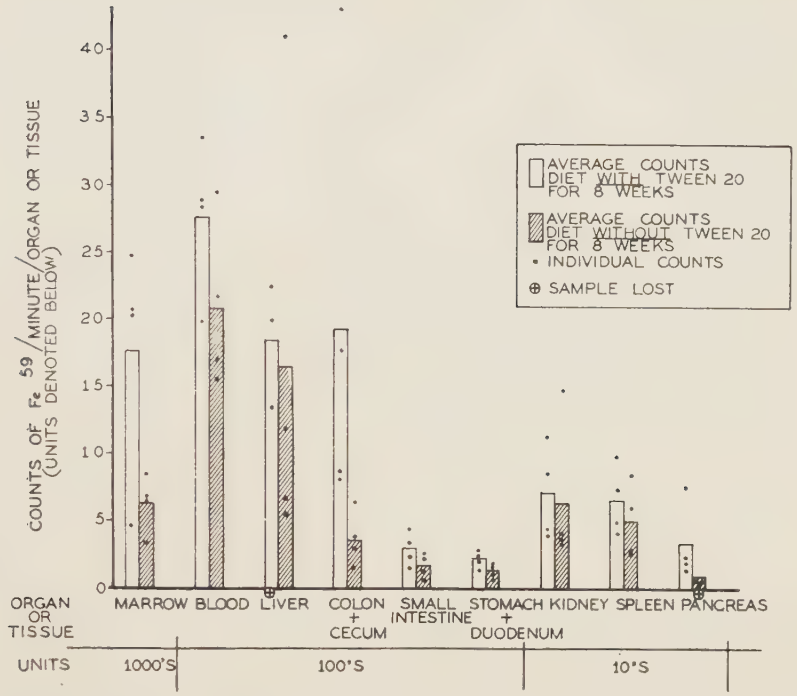

FIG. 1. Exp. 1. Summary of distribution of Fe^{59} in organs and tissues of Tween-fed and control hamsters 3 days after receiving 344000 counts of Fe^{59} by stomach tube. Individual and average counts are expressed as counts per min. per total organ or tissue and are corrected for decay.

allowed to incubate in a warm place for about 2 hours and was then baked at 200°C for 30 minutes. The resulting bread was sliced and air dried at room temperature. It was then ground and the remaining ingredients were mixed with the pulverized ration.

After receiving the rations for varying times equal groups of control and Tween fed hamsters were given concurrently an appropriate single dose of Fe^{59} by stomach tube.‡ The animals were then transferred to individual metabolism cages and urine and feces were collected daily. Seventy-two hours later the animals were sacrificed and specimens of various organs were obtained, weighed and prepared for counting as were the urine and fecal samples. In the first experiment carrier iron was added to each flask containing a sample to make a total of about 10 mg of iron. Wet ashing and electroplating of the sample were then carried out by the method of Peacock and Evans(3) as modified by Huff(4). The plated samples were counted using a lead-shielded end-window Geiger-Muller tube (Tracerlab TCG-2). The samples from the second and third experiments were counted directly in small plastic tubes using a well type scintillation counter. In the second and third experiment each animal was perfused with 100 ml of 0.86% NaCl before the tissue samples were taken to minimize blood contamination. Precautions were observed in each experiment to recover feces adhering to the cages. When residual radioactivity in carcasses was determined in Exp. 3, care was taken to include intestinal contents and fecal material about the anus in the excreta compartment. The parts of the intestinal tract were washed in iron free NaCl before counting. The carcasses were ground

‡ The Fe^{59} was supplied by Oak Ridge National Laboratory, and was prepared by neutron bombardment of electromagnetically separated Fe^{58}. Before use, the $FeCl_3$ solution was neutralized by addition of saturated sodium citrate with chlor-phenol red as an indicator.

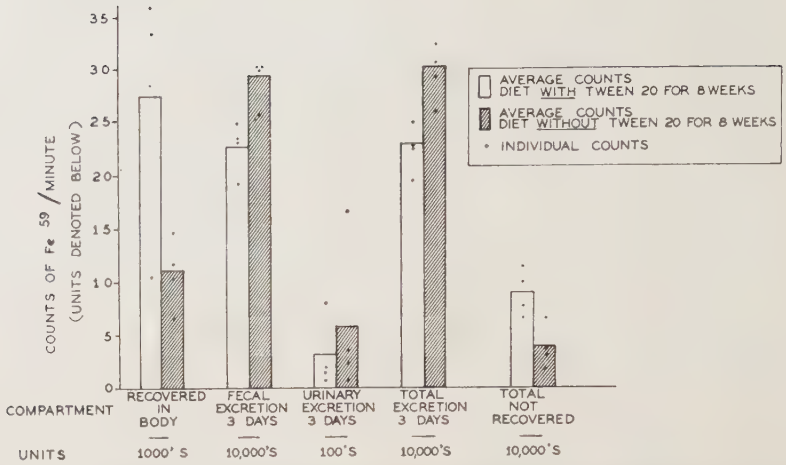

FIG. 2. Exp. 1. Summary of absorbed and excreted Fe^{59} in Tween-fed and control hamsters 3 days after receiving 344000 counts of Fe^{59} by stomach tube. Results are corrected for decay.

in a meat grinder, dissolved in 2 N NaOH, heated at 60°C for 2-3 days, and aliquots were then removed for counting. Samples of liver, spleen and cecum were wet ashed and analyzed for total iron along with appropriate controls in Exp. 3 by the method of Barkan and Walker(5) with ascorbic acid substituted for hydrazine sulfate as the reducing agent.

Small tissue samples from each animal were fixed in formalin, imbedded in paraffin and stained for iron by the Prussian blue reaction. Hematoxylin and eosin stains were also examined from the liver, spleen, pancreas, kidneys, mesenteric lymph nodes and the various parts of the gastrointestinal tract.

Results. Diet consumption and weight gains of the 2 groups of hamsters were essentially the same in each experiment. Intermittent diarrhea was apparent in both groups but was more common in the Tween fed animals. Fig. 1 summarizes the individual and average radioactivity measured in Exp. 1 from the tissues of 4 pairs of hamsters 3 days after each animal had been given Fe^{59}, equivalent to 344000 counts per minute or about one μc and about 1.5 μg, and after they had received the respective diets for 8 weeks. Although considerable individual variation was encountered, there was a definite trend for the animals receiving the Tween to show more radioactivity in the marrow, blood, liver and large intestine 3 days after the test dose of Fe^{59} had been given. In each instance the counts represent the calculated amount of radioactivity in the total organ or tissue.§

Fig. 2 demonstrates that the greater amount of Fe^{59} apparent in the tissues of the Tween fed animals was associated with a decreased excretion of Fe^{59} and that there was more unrecovered in the Tween fed animals than in the controls, possibly due to excess Fe^{59} present in the parts of the carcass not analyzed or due to incomplete recovery of excreted Fe^{59}.

Although the daily fecal and urinary Fe^{59} excretion have not been illustrated, individual results indicated that the majority of the Fe^{59} was excreted during the first 48 hours. It is noteworthy that very little of the Fe^{59} was excreted in the urine, an observation which agrees with previous studies of iron metabolism(6,7).

In the second experiment 2 groups of animals were studied after they had been fed the respective rations for 10 weeks. The experiment differed from the first in that the Tween was added to the ration rather than being substituted for a portion of the fat in the ration, the tissues were perfused before samples were obtained for counting and the

§ For these calculations the theoretical total bone marrow was considered to be 1%(4) of the body weight and blood was considered to be 5% of the body weight.

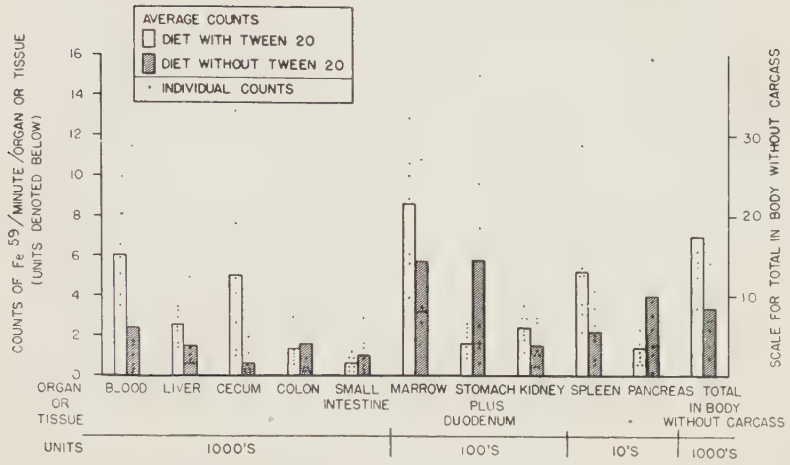

FIG. 3. Exp. 2. Summary of individual and average counts of Fe^{59} in organs and tissues of Tween-fed and control hamsters 3 days after receiving 375000 counts of Fe^{59} by stomach tube. Results are corrected for decay and are expressed as counts per min. per total organ or tissue.

more direct gamma counting methods were employed. Each animal received 2 ml of the iron solution containing 375000 counts per minute or approximately 0.5 μc in about 1.0 μg of iron, and each was sacrificed 72 hours later. Fig. 3 illustrates the same trends noted in the first experiment. The blood, marrow, liver and cecum of the Tween fed animals contained more of the ingested dose than the same organs of the control animals. Two factors complicated this experiment: 1) occasional control animals showed unusually high Fe^{59} values which detracted from the statistical competence of the data. 2) It was impossible to obtain a good measure of carcass Fe^{59} since adherence of fecal material about the anus caused some of the residual carcass counts to be very high and the excreta counts to be correspondingly low. Measures to avoid this complication were taken in the third experiment.

The third experiment was similar to the second except that the animals had been fed the respective rations for 20 weeks before the tracer dose of about one million counts of Fe^{59} containing about 1.0 μc and about 1.5 μg of iron was administered orally. In this experiment only the organs or tissues which had shown consistent differences in the first 2 experiments as well as the spleens were counted separately. A measure of the remaining radioactivity in the animal was obtained by determining the counts on the residual carcass. In Fig. 4 it is apparent that with one or 2 exceptions quite consistent differences were observed between the organs of the Tween fed animals and those of the control animals. The cecum showed the greatest contrast between the 2 groups; the cecums of the Tween fed animals averaging almost 40 times the amount of Fe^{59} as compared to the cecums of the control animals. The other organs and tissues of the Tween fed hamsters tested averaged about 2 times the quantity of Fe^{59} found in the controls. Similarly the residual carcasses of the animals receiving Tween contained about 6 times as much Fe^{59} as the control carcasses. The overall balance of absorbed and excreted Fe^{59} is also shown in this chart. Here again it is apparent that there was a definite difference between the 2 groups with the Tween fed animals showing more absorption and less excretion. The group receiving Tween 20 had an average of about 8% of the ingested Fe^{59} unaccounted for in the overall balance, whereas there was no such deficit in the control group. It seems probable that most of this deficit was due to incomplete recovery of excreted Fe^{59} in 2 of the Tween fed animals. A small part of the deficit was undoubtedly due to the samples of the cecum and other organs removed for histological study, since these samples constituted a larger proportion of the ingested tracer dose in the hamsters

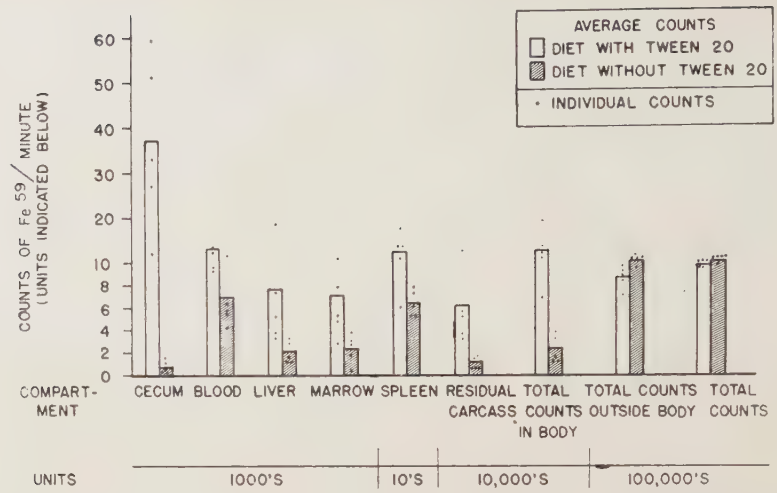

FIG. 4. Exp. 3. Summary of individual and average counts of Fe^{59} in certain organs and in other compartments 3 days after Tween-fed and control hamsters received 1 million counts of Fe^{59} by stomach tube. Results are corrected for decay and are expressed as counts per min. per total organ or tissue or per total excreta.

fed the Tween ration. In any case addition of these missing counts on either side of the balance sheet would not alter the conclusions to be drawn.

Hemoglobin and hematocrit determinations on the blood of each of the animals of this experiment at the time of sacrifice demonstrated no anemia and no differences between the 2 groups. Therefore anemia does not appear to have caused the increased iron absorption in the Tween fed group.

Histological study of the Prussian blue stained tissues of the 2 groups of animals in each experiment revealed a consistent increase in stainable iron in the cecums, large intestines, livers, and mesenteric lymph nodes of the animals receiving Tween 20.

Furthermore, total iron analyses on the spleens, livers, and cecums of the animals of Exp. 3 revealed an average of about 5 times as much iron in the cecums of the Tween fed animals as in the cecums of the controls and about 1½ as much in the livers of the Tween fed animals as in the livers of the controls. There was no significant difference in the iron content of the spleens of the 2 groups (Fig. 5).

The stainable iron in the cecums was found in the epithelial cells and in the macrophages of the stroma of the mucosa (Fig. 6). The increased visible hepatic iron was largely in parenchymal cells about the portal triads with lesser quantities in the Kupffer cells and in the macrophages of the portal triads (Fig. 7). Occasional livers of the Tween fed groups of hamsters showed early cirrhosis (Fig. 8) but the marked cirrhosis accompanying the iron deposition reported by Eagle(1) in younger hamsters receiving polyoxyethylene derivatives for longer periods of time was not observed. No appreciable inflammation of the gastrointestinal mucosa was observed

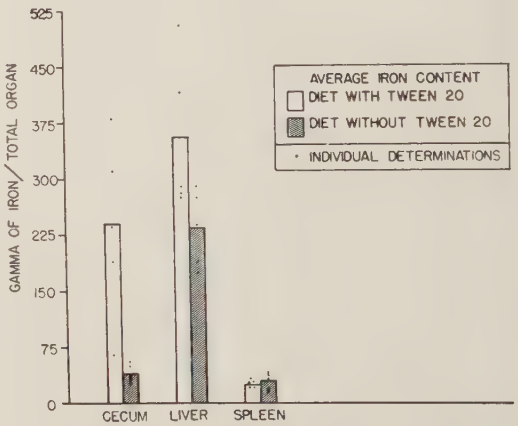

FIG. 5. Exp. 3. Results of chemical analysis of selected organs for total iron expressed as gamma of iron per total organ.

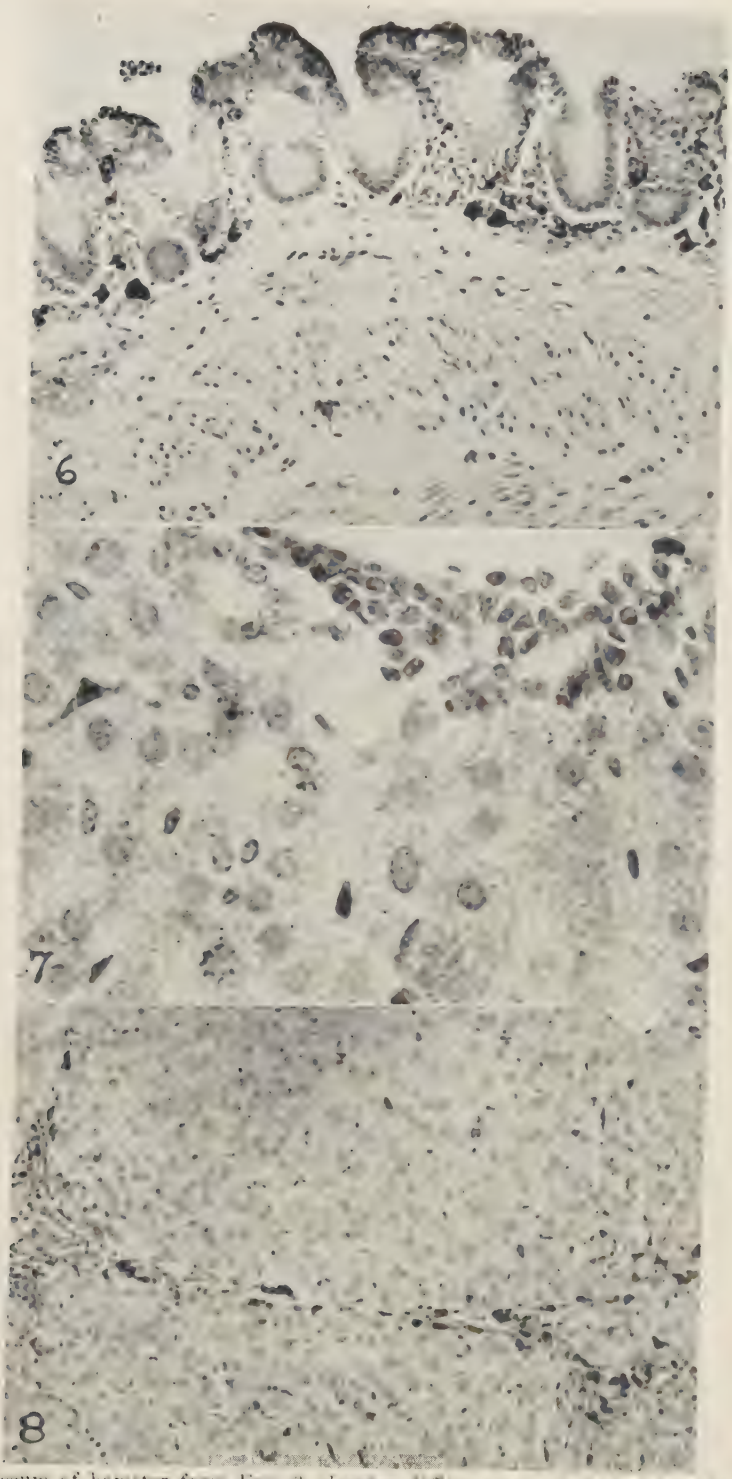

FIG. 6. Cecum of hamster from Exp. 3, showing diffuse accumulation of iron in epithelial cells nearest the lumen and in macrophages in stroma of the mucosa. ×193.

FIG. 7. Liver of hamster from Exp. 3 stained by Prussian blue reaction. Dark granules in the parenchymal cells, Kupffer cells and periportal macrophages represent stained iron. ×634.

FIG. 8. Liver of hamster from Exp. 3 showing early cirrhosis and abundant iron positive granules in macrophages of the fibrous tissue and in the Kupffer cells. ×150.

in any of the animals receiving the Tween ration.

Comment. The results of these 3 experiments indicate that the presence of 5% Tween 20 in the diet of the golden hamster results in a consistent increase in the gastrointestinal absorption of iron. This apparently increases with the time the animal is maintained on the Tween containing ration. It occurs when the Tween 20 is added to the ration or when it is substituted for a part of the fat in the ration. It is accompanied by an increase in histologically demonstrable iron in the cecum and to a lesser extent the rest of the large intestine as well as the liver and mesenteric lymph nodes. The increase in hepatic and cecal iron has also been demonstrated by chemical analysis. The absence of anemia in the Tween fed hamsters indicates that this well known mechanism(7) for increasing iron absorption from the gastrointestinal tract was not responsible for the differences between the 2 groups.

The largest and most consistent differences in Fe^{59} content between the experimental and control animal tissues 3 days following the administration of the tracer dose of iron by stomach tube occurs in the gastrointestinal tract, bone marrow and blood. This is noteworthy since these are the tissues concerned in iron absorption, erythrocyte formation and erythrocyte circulation.

Although the mechanism by which Tween 20 increases iron absorption from the gastrointestinal tract is not yet clear, the results of this study would suggest that the excess iron is being absorbed largely in the cecum. The cecums enlarge in the Tween fed animals and their contents become more fluid. Associated with this is a tendency toward diarrhea, but no inflammation of any part of the gastrointestinal tract was observed histologically in the Tween fed animals. The similar weight gains and diet consumption of the 2 diet groups in these relatively short feeding periods fail to implicate an inherent toxicity of the 5% Tween ration or any gross malnutrition in the mechanism. Whether the emulsifying agent acts mainly on the epithelium to increase its permeability or whether it converts gastrointestinal iron into a more absorbable physical or chemical state remains to be investigated.

The results of the experiments suggest but do not prove that one mechanism by which the usually well-regulated gastrointestinal absorption of iron can be increased is by extending the area of absorption from the small intestine, where the majority is usually absorbed(7-9), to the large intestine.

Summary. In 3 experiments adult golden hamsters fed a fortified bread ration containing 5% polyoxyethylene sorbitan monolaurate (Tween 20) for from 8-20 weeks showed a consistent increase in gastrointestinal absorption of a test dose of radioactive iron (Fe^{59}) during the subsequent 3 days. Increases in the isotope were noted especially in the cecum and large intestine, the blood, bone marrow, and liver. Concomitantly less radioactive iron appeared in the excreta of the Tween fed animals than in the excreta of control animals. This evidence of increased iron absorption in the hamsters was accompanied by the presence of iron pigment in the cecal mucosa, mesenteric lymph nodes and liver and by an increase in chemically demonstrable iron in the cecum and liver.

The authors wish to acknowledge the technical assistance of Mrs. Marjorie Schroeder and Mrs. Kathryn Soules. The photomicrographs were taken by Mr. Jean Crunelle, chief photographer, University of Chicago Clinics.

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Intradermal Test for Determining Resistance to Infectious Canine Hepatitis Virus.* (21043)

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Studies on infectious canine hepatitis virus (ICH) are limited, in part, because of a) the inability to use a convenient small laboratory animal and b) complement-fixation tests are not always a reliable index of past infection. Many resistant dogs may show a negative complement-fixation test. The costly and elaborate procedure for raising disease-free animals is impractical for most laboratories. Street dogs are barred from routine use because of natural infection and resulting immunity.

In the course of clinical and experimental studies with ICH virus, it was found that the intradermal injection of apparently normal dogs with lymphoid tissue from an infected animal caused a marked reaction in some cases. The relationship of this reaction to resistance to ICH virus was, therefore, investigated.

Materials and methods. Virus strains. Two strains of infectious canine hepatitis virus were used in the study. One strain, designated 9868, was obtained from Dr. H. R. Cox and the other, 21V33, from Dr. C. J. York. Both strains were passed through at least 2 serial passages in dogs before use. Inocula used were 30% suspensions of infective liver. Intravenous injections were used and dogs killed on the third post-inoculation day by an overdose of sodium pentabarbital. Several dogs were used for each passage and one chosen that demonstrated a sharp temperature rise and simultaneous clinical symptoms of the disease. The liver was harvested under sterile conditions and placed in a previously weighed Waring blendor flask. The flask and liver were weighed and the weight of the liver alone determined. Sufficient buffered saline (pH 7.2) was added to make a 30% suspension of liver and the mixture homogenized for 3 minutes. The homogenized material was distributed in 2 ml amounts to sterile ampoules, sealed, shell frozen and stored in a dry ice chamber. Prior to intravenous injection, an additional 1 ml of sterile saline was added to the thawed suspension. *Street dogs* obtained from a stock supply were used without regard to age, size or sex. The great variation in animals led to the assumption that this sampling would represent a cross section of an average urban canine population. One hundred and twenty-four dogs were used in the series. Dogs were maintained in community pens except for challenge experiments, when they were placed in individual cages. *Complement-fixation tests.* A liver antigen was used in all tests. This was prepared from the liver of a dog experimentally infected with strain 9868 (Lederle). The liver was homogenized with sufficient buffered saline to make a 30% suspension. The mixture was centrifuged at 2000 r.p.m. for 15 minutes. The supernatant was distributed to 1 liter round-bottom flasks and shell frozen in a dry ice-alcohol bath. After immediate thawing, the mixture was recentrifuged at 2000 r.p.m. for 30 minutes. The resulting supernatant was distributed in 50 ml amounts, formalized (0.4%) and shell frozen. Lyophilization was performed after storage for 24-72 hours at −20°C. The dried material was ground with a mortar and pestle and stored in brown bottles at 4°C. This material

* Aided in part by a grant from the Hendrick's Research Fund.

was resuspended at a ratio of 100 mg of dry material to 1 ml of saline immediately before use. Complement-titrations were done in the presence of antigen and antigen titrations were done against known positive sera. Approximately 2 units of antigen were then used in subsequent tests. Blood samples were obtained at the same time skin tests were performed, serum separated and stored at –20°C until the tests were done. Sera were diluted 1-4 and inactivated at 57°C for 30 minutes prior to use. Positive and negative serums were included in each test as controls. Titers of 1-8 and above were considered positive.

Skin test antigens. Cervical lymph nodes were used for the preparation of skin test antigens. Dogs in the acute phases of infectious hepatitis were killed. The submandibular nodes were removed and examined for evidence of lymphadenopathy. Only glands that were enlarged and edematous were used for antigen production. The node was stripped of fat, weighed and placed in a stainless steel blendor. Sufficient saline was added to make a 1:10 suspension. The mixture was homogenized for 5 minutes and the material allowed to stand for 5 minutes. The decanted fluid was used as an antigen after formalizing to 0.2%. One-tenth milliliter was injected intradermally as a test dose. Reactions were read 24 and 48 hours after injection. Definite raised indurated areas at the site of injection with or without surrounding erythema were considered positive and slight or absent indurations classified negative.

TABLE I. Comparison of Skin-Test Reactions and Complement-Fixation Reactions for Infectious Canine Hepatitis Antibodies.

No.	Skin reaction		C-F positive*
	Positive	Negative	
18	12	6	2/10
10	6	4	1/6
7	1	6	0/1
3	3	0	0/3
6	3	3	1/3
2	1	1	0/1
2	0	2	—
3	2	1	1/2
5	3	2	1/3
11	6	5	1/5
7	3	4	0/2
17	5	12	1/5
Total 91	45	46	8/41

* No. positive/No. showing positive skin reactions.

TABLE II. Challenge of 35 Dogs Showing Positive and Negative Skin Reactions with ICH Virus.

No.	Skin reaction	Method of challenge	Response (Positive/No. challenged)
16	Positive	I.V.*	1/16
12	Negative	I.V.	11/12
5	"	I.O.	5/5
2	Doubtful	I.V.	1/2

* I.V. = intravenous; I.O. = intraocular.

Results. A total of 91 animals were examined by the intradermal skin test. Eighty-two of these animals were also examined for complement-fixing antibodies. Of the dogs showing a negative skin test, all but one were negative in the complement-fixation test.

As seen in Table I, only a few animals showing positive skin reactions were also positive in the complement-fixation test. Eight of 41 dogs with positive skin tests or 19.5% had positive serologic reactions. Although the formation is not listed, dogs that had been experimentally infected with virulent virus developed complement-fixing antibodies.

Table II shows the results of challenge of both negative and positive reactors with virulent infectious canine hepatitis virus. Seventeen negative reactors were challenged. Of this group, one animal reacted doubtfully and 16 developed infectious hepatitis. In a group of 16 dogs with positive reactions, 14 were resistant to experimental infection. An additional animal with a doubtful reaction was positive to challenge and another was negative.

The effect of prophylactic doses of infectious canine hepatitis antiserum on the subsequent skin test is shown in Table III. Twelve animals were given serum before the skin test and 10 showed positive reactions. In 59 animals not receiving serum, only 28 were positive to the intradermal test. Passive immunization apparently results in rendering the animal sensitive to skin test antigen. The subsequent natural disease ratio is also shown. The only conclusion that can be assumed from the latter is that animals showing a positive ICH skin test reaction probably carry specific antibodies for other common diseases of the dog as leptospirosis, canine distemper and broncho

TABLE III. Relationship of Serum Prophylaxis to Infectious Canine Hepatitis Skin Test and Subsequent Incidence of Disease.

No.	Serum admin.	Skin test		Subsequent disease ratio	
		Positive	Negative	Pos. skin	Neg. skin
12	Before skin test	10	2	2/10	1/2
59	No serum	28	31	8/28	19/31

pneumonia. Incidence of these infections in negative contact animals indicates that exposure was common.

Discussion. Infectious canine hepatitis (ICH) is well known as a common disease of the dog and fox, but other species are not susceptible either naturally or experimentally (1-3). Basically, the agent is an interesting subject for virus study because of its resistance to adaptation to other species and high specificity for endothelial structures(4). Also, it is of growing importance as a natural pathogen of the canine(5).

There is insufficient evidence at present to consider an interdermal test as a possible routine diagnostic measure for infectious canine hepatitis. Extensive field trials will be necessary to evaluate its efficiency fully. It is probable in cases of question as regards prophylactic infectious canine hepatitis serum therapy, that all animals showing a negative or questionable reaction should be considered susceptible to the disease. Positive reactors in all probability are immune, providing recent serum prophylaxis has not been given.

The test may remove the necessity of challenging individuals in a litter to determine the immune status before using them for research on infectious hepatitis. The usual procedure presently followed is to challenge one member of a litter and assume the status of the remaining puppies according to the challenge result.

The present complement-fixation test is inadequate as a diagnostic procedure. Apparently, many naturally recovered animals carry insufficient titers for recognition by the test. Also, the complexity of the antigen preparation and lack of standardization lessens its value and effectiveness as a routine diagnostic test. The intradermal test, if as effective as preliminary study indicates, though not perfect, would far surpass the complement-fixation test as a routine in both experimental and clinical work. Future studies are contemplated in order to evaluate the anamnestic effect of the skin test on complement-fixing antibodies.

Summary. An intradermal test for detecting resistance to infectious canine hepatitis is described. Sixteen of 17 animals showing negative tests were susceptible to the disease. Fourteen of 16 positive reactions were resistant to experimental challenge. The test apparently has a much greater sensitivity than the complement-fixation test.

The technical assistance of Mr. Sam Martin in carrying out many details associated with this work is appreciated.

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Propagation of Bunyamwera, West Nile, Ilheus, and Br I Viruses in Human Cells in Tissue Culture.* (21044)

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The data to be presented demonstrate the sustained propagation of West Nile, Egypt 101, Ilheus, Bunyamwera, and Br I viruses in tissue cultures of human normal lymph nodes, and suggest that prolonged propagation of Bunyamwera virus in these human cells has altered its pathogenicity for mice. These studies also indicate that these viruses have little cytopathogenic effect under these experimental conditions.

Materials and methods. Viruses. Bunyamwera, West Nile, Egypt 101, and Ilheus viruses are mosquito-borne viruses capable of causing encephalitis in man and of inhibiting growth of certain mouse tumors. Egypt 101 is a recently isolated virus(3) which appears to be serologically identical with West Nile virus, but differs from the original isolate in its pathogenicity for man(11,12). Detailed literature surveys are available elsewhere(4,13). Br I virus was isolated from mouse brains by Dr. Hilary Koprowski during studies of intracerebral heterologous tumor growth (personal communication). Prior to growth in tissue cultures, all viruses were maintained by intracerebral passage in mice. Identity of Ilheus, West Nile, and Egypt 101 viruses was established before and during the tissue culture studies by neutralization tests with known specific antisera (neutralization indices of 100 or greater). Br I virus was recently obtained from Dr. Koprowski and serologic identification was not attempted. Neutralization studies with Bunyamwera virus failed to show neutralization of more than one log using 2 sera supplied by other laboratories or sera from rabbits repeatedly inoculated with this virus in our own laboratory. The virus is, nevertheless, considered to be Bunyamwera because the original stock from which these studies were initiated was so designated, and because its infectious characteristics for the mouse (described in text) are in agreement with the original descriptions(8) and have remained constant throughout studies of this virus in mice at this institute. *Tissue cultures.* The cells were grown from histologically normal lymph nodes obtained by therapeutic node dissection from patients with various types of cancer. The roller slide technic was used. This entailed the embedding of tissue fragments in chicken plasma clot on a free cover slip which was then placed in a roller tube with 2.0 ml of a nutrient medium consisting of 45% Z 16 balanced salt solution(5), 50% cell-free ascitic fluid from humans with cancer, and 5% chick embryo extract. Cultures were incubated in roller drums at 37°C for 6 to 14 days prior to the introduction of virus (a single exception was incubated for 28 days prior to inoculation) and were not used for virus inoculation unless cellular outgrowth occurred. The cells were chiefly fibroblastic. Lymphocytes were common in young cultures, but rare after 10 days. It has therefore been assumed that the cells which supported the virus growth were fibroblasts. Cultures were fixed and stained at the end of each study. Cytologic study of living cultures was not attempted. *Virus technics.* For primary inoculations 0.2 ml of 1.0% virus-infected mouse brain was added to 3 or more tubes of a tissue culture, and an equal number of control tubes of the same culture received normal mouse brain suspension. The initial virus dilutions thus became 10^{-3}. For subsequent passages, supernatant fluids of the 3 replicate tubes were pooled and 0.2 ml was transferred undiluted to new cultures containing 1.8 ml of nutrient solution. Cultures were re-fed at intervals of 4 to 10 days, thus causing a further 10-fold (or greater) dilution of the original mouse-brain inoculum. Viruses were titered in 18-20 g female white

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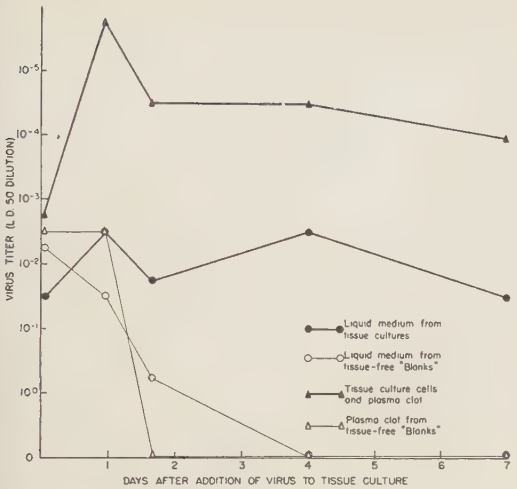

FIG. 1. Demonstrating rapid disappearance of Bunyamwera virus when incubated in the absence of cells, in contrast to persistence of virus in cells and in the supernatant fluid from tissue cultures.

Swiss (Banks) mice by intracerebral inoculation of 0.03 ml of serial 10-fold dilutions of tissue culture fluid (or a triturated suspension of cells and clot in one experiment). Three mice were used per dilution. LD_{50} titers were calculated by the Reed-Muench method(6). If undiluted fluid (10° dilution) contained some virus, but less than an LD_{50}, it is plotted in the figures midway between 0 (no virus demonstrable) and 10°. Control and infected cultures were treated identically through all re-feedings, passages, and titrations.

Results. Bunyamwera virus. Proofs of propagation. When Bunyamwera virus was added to a tissue culture "blank" consisting of glass, plasma clot, and nutrients, but without any cells, detectable virus decreased rapidly in the first 24 hours, and no virus was detectable when tested at 4 days or thereafter (Fig. 1). In marked contrast, in one experiment, Bunyamwera virus was consistently maintained within a single tissue culture (re-fed at weekly intervals) for 94 days. Another similar study was maintained for 42 days. The virus was still present at the end of the studies when the cultures were fixed for cytologic examination. The similarity of form of the virus propagation curves in the parallel studies of tissue and of culture medium (Fig. 1) suggests that the titrations of virus in the medium reflect the amount of virus within the cells. The virus titers in the cells (plus plasma clot) average about 2.5 logs higher than in the liquid medium. That this difference represents the concentration of virus in cells, rather than in the clot, is indicated by the fact that there was no more virus in the clot than in liquid medium in the tissue-free "blanks".

By consecutive transfer from culture to culture, Bunyamwera virus was maintained through a total of 20 separate tissue cultures for a total lapsed time in culture of 159 days. The virus (in tissue culture medium) was stored in a dry-ice box on 7 occasions between tissue culture passages for a total period of 213 days. At no time during this passage study was the virus propagated in any tissue except the human lymph-node cells in tissue culture. On one occasion the tissue culture medium containing virus was Seitz filtered prior to transfer to new tissue cultures. Inoculation of mice with medium from the normal-mouse-brain-control tissue cultures of each passage caused death of only 3 out of a total of 315 mice.

Computation of the dilution of the original mouse-brain inoculum during these studies indicates that at the end of the 20th consecutive tissue culture passage the *original* inoculum had reached a dilution of $10^{-26.0}$. In contrast, the LD_{50} intracerebral titer of the original virus inoculum was $10^{-7.5}$. This quadrillion-fold difference must be due to virus propagation. In the study in which virus was maintained in a single continuous culture for 94 days, the dilution of the original inoculum by the time this tissue culture was infected (18th tissue culture passage) was 10^{-23}, and by the time the culture was fixed (after 12 re-feedings) the original inoculum had been diluted to 10^{-35}. In a single culture in which virus was maintained for a period of 42 days, titrations were performed on 18 occasions. These titers fluctuated between 10^{0} and $10^{-3.5}$ without apparent relationship to total lapsed time in culture or to time of re-feeding of the cultures. Further evidence of the propagation of Bunyamwera virus was provided by 3 studies in which the virus titer in culture medium was determined

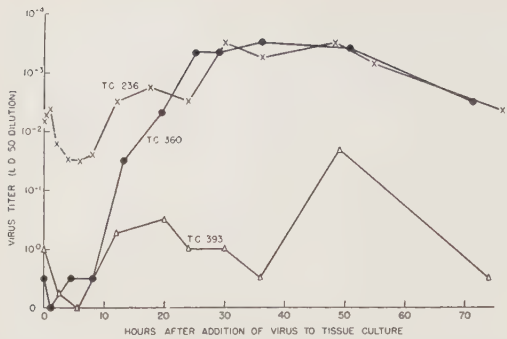

FIG. 2. Demonstrating increasing virus titers in culture medium during 3 days following addition of Bunyamwera virus to tissue cultures.

at frequent intervals following inoculation (Fig. 2). During the first few hours after virus inoculation, there was a drop in titerable virus. By 12 hours there was an increase, and peak titers were reached between 18 and 24 hours. Examination of these curves (especially tissue cultures 236 and 393) suggests that the infective cycle for this virus is approximately 18 to 24 hours. In culture 360, there was a greater than one-thousand-fold increase in virus titer during the 24 hours following inoculation.

In addition to the 20 serial passages already discussed, Bunyamwera virus was transferred in several sublines in human lymph node tissue cultures for various types of special studies. In all, this virus was inoculated into 36 different tissue cultures (*i.e.*, lymph nodes from 36 patients). Virus propagation could not be demonstrated in 6 (17%) of these attempts. These failures were not due to poor cellular growth or to inadequate virus inoculum, and are still unexplained. It seems most probable that virus could not be demonstrated in these 6 cultures because it had not propagated, but it is conceivable that it had mutated to a form which was avirulent for mice.

Adaptation of Bunyamwera virus in tissue cultures. There is some evidence that during these passages in human cells there was adaptation of Bunyamwera virus to this new host. This is suggested by a progressive change in the picture of disease produced in mice. The original mouse-brain-passage Bunyamwera virus killed mice in 3 to 5 days even when inoculated at the minimum lethal dose. Paralysis occurred rarely and lasted only a few hours before death. Usually death occurred suddenly with few signs of sickness other than a brief terminal convulsion. After the virus had been through 10 passages (58 days) in human cells, it caused a more chronic illness of mice. Mice injected with this passaged virus frequently died 10 to 14 days after inoculation, and it became common for paralysis to persist for 2 or more days before death; and a few mice recovered after several days of paralysis. The prolonged period of illness and prolonged incubation time with the tissue-culture-passaged virus was observed even when mice were inoculated with as much as 1000 times the LD_{50}, indicating that the change in character of the disease in mice was a result of change in pathogenic character of the virus rather than a dose phenomenon. Fig. 3 summarizes the data which suggest virus adaptation. In this figure are included results from all cultures tested between days 2 and 7 after virus inoculation. From one to 4 samples were studied during this period in each passage. Studies earlier than day 2 were excluded because they might measure non-propagating virus or sub-maximal proliferation. Studies later than day 7 were excluded because they were available for only a few cultures. The lowermost curve presents the virus titers, as measured by lethality for mice. These titration data are incomplete because several cultures were tested undiluted only; however, it is apparent that there was no consistent tendency toward increased or decreased virus titer (as measured in mice) during serial passage in culture. The remaining 2 parts of Fig. 3 illustrate the progressive change in pattern of the illness produced in mice by the virus during serial tissue-culture-passage. For these curves only those mice inoculated with undiluted medium were considered. The middle section of the figure illustrates the progressive prolongation of average survival time of mice inoculated with virus grown in successive tissue culture passages. In order to permit a computation of average survival times for each passage, those mice which were alive at the end of the 2-week observation

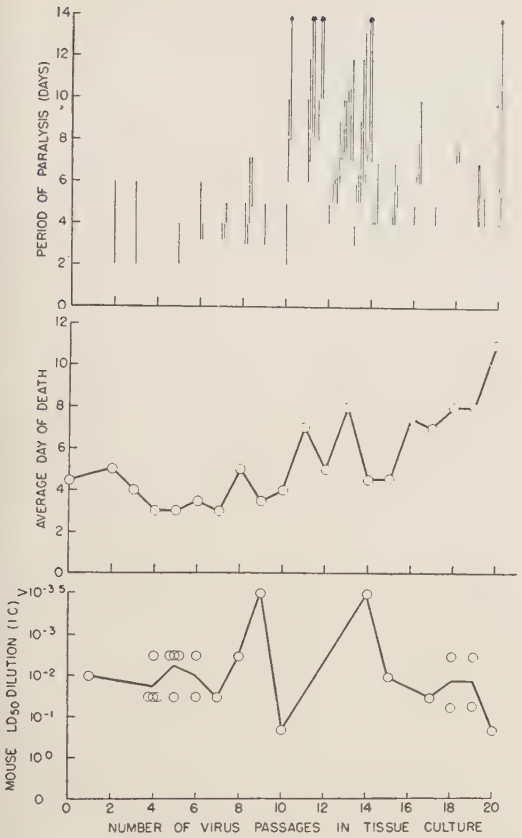

FIG. 3. Demonstrating change in type of illness produced in mice by Bunyamwera virus during course of prolonged passage in tissue cultures of human cells. Lower portion of figure shows virus titers as measured in mice. Middle portion shows progressive increase in survival time of mice inoculated with undiluted tissue culture medium from the serial passages. Upper portion shows duration of paralysis in individual mice (arrows indicate recovery from paralysis).

period were included in the calculation as if they had died on day 15. The uppermost part of the figure indicates the prolonged paralysis frequently seen in mice inoculated with high tissue-culture-passage virus. Each line indicates the duration of paralysis in one mouse. The paucity of lines on the left half of this chart reflects the rarity of prolonged paralysis from early passage virus. It can be seen, by comparison of these curves that the changes in the pattern of illness in the mice are not a function of virus titer. For example, in passages 4 through 7 titers are almost identical with the titers in passages 17 through 19, but average survival time of mice is 3 days and 8 days respectively for these same groups.

That these changes were due to adaptation is also suggested by the fact that when "adapted" virus (13th tissue culture passage), was carried through 3 mouse passages, it regained the original pathogenic pattern of rapid death preceded by convulsions or very brief paralysis (even at the minimum lethal dose).

Cortisone and virus growth in culture. Because of evidence that cortisone treatment increases susceptibility of mice to Bunyamwera virus(9), and has similar effects on other viruses in various hosts(1,2,7,9), the effect of cortisone acetate and dehydro-cortisone acetate (compound F) on the growth of this virus in these tissue cultures was studied. Cortisone was used in a final concentration in the culture medium of 5, 50, and 500 γ/ml. Compound F was used at 50 γ per ml. Control cultures contained the same solvents and suspending agents as were used for the steroids. There was no significant effect upon virus propagation as judged by serial titration of virus in the culture medium (Fig. 4). These results suggest that cortisone affects host susceptibility to virus infection indirectly, rather than by an effect upon the parasitized cell. Interpretation must be tentative, however, because it is possible that the acetate form of these steroids is not active at the cellular level, and could not be broken down by the fibroblastic cells.

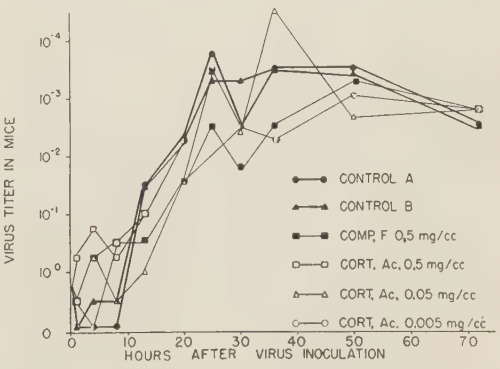

FIG. 4. Demonstrating failure of cortisone acetate or dehydrocorticosterone acetate to influence rate or degree of virus propagation in cells in tissue culture.

FIG. 5 (top). Normal appearing fibroblastic type cells from human lymph node in tissue culture. Culture was 54 days old and had been inoculated with Bunyamwera virus 42 days before fixation. Virus was still present at end of experiment. Stained by Jacobsen's technic with May-Gruenwald and Giemsa stains. T.C. No. 278.

FIG. 6 (middle). Normal-mouse-brain control for culture in Fig. 5.

FIG. 7 (bottom). Same as Fig. 5 except that this culture had supported growth of Egypt 101 virus for 39 days.

Cytopathology. There was a surprising lack of cell damage in virus-infected tissue cultures, indicating that Bunyamwera virus is able to grow in human fibroblasts without killing the cells, or that the rate of cell proliferation is equal to the rate of cell destruction. Areas of degenerating cells were occasionally seen, but did not appear more common than in the normal-mouse-brain control series of tissue cultures. There was poorer cell growth in many virus-infected cultures than in parallel controls, suggesting inhibition of cell propagation, but this difference was not sufficiently consistent to be convincing, and quantitation of cell growth was not attempted. Fig. 5 shows normal fibroblastic cells after 42 days of virus proliferation. Fig. 6 is the normal-mouse-brain control of the same culture.

West Nile viruses. The Egypt 101 isolate of West Nile virus was inoculated into 12 lymph node cultures. Virus propagated, or at least persisted, in 7 (58%). The reason for the failures was not determined, but it was not absence of cell growth nor absence of virus in the inoculum. The longest serial passage of Egypt 101 virus was through 4 consecutive tissue cultures for a total of 79 days. The titer of the original inoculum in this study was 10^{-6} (*i.e.* mouse LD_{50}). The dilution of the original mouse-brain inoculum produced by 4 culture passages and 12 re-feedings was 10^{-17}, indicating that at least a 10-billion-fold increase of virus had occurred. The tissue-culture virus was Seitz filtered prior to the fourth passage. In another culture, not included in the above series, Egypt 101 virus was demonstrable for 39 days. There were 9 re-feedings during this period, thus resulting in 10^{-11} dilution of the original mouse-brain inoculum. This indicates a 10000-fold increase of virus in this single tissue culture, since the original inoculum for this culture titered 10^{-7}.

Smithburn's original isolate of West Nile virus was inoculated into 4 cultures and prop-

agated in 2. There is no apparent explanation for the 2 failures, and it is of interest that both of the cultures which failed to support growth of Nest Nile virus did support growth of Bunyamwera virus in parallel studies. The longest persistence of West Nile virus was 37 days. During this period the culture was re-fed 5 times, resulting in dilution of the original inoculum to 10^{-7}. There was no consistent cytopathology attributable to either the Egypt 101 isolate (Fig. 7) or the original isolate of West Nile virus.

Ilheus virus. Ilheus virus was inoculated 10 times into lymph node cultures and propagated, or at least persisted for several days, in 7. The longest successful serial passage was through 4 cultures for a total of 163 days in culture, and a final dilution of the original mouse brain inoculum to 10^{-27}. The original inoculum had a titer of 10^{-6}. Each of these 4 serial passages was maintained (with persistence of viable virus) for over 30 days. No convincing evidence of cytopathology was seen.

Br I virus. Br I virus was inoculated into 6 cultures of human nodes and propagated in all. Four of these were in serial passage for a total duration of 135 days. The original inoculum, which titered $10^{-7.2}$, was diluted to 10^{-23} by these passages and re-feedings. This implies at least a 10^{15}-fold increase in virus in tissue culture. The virus was Seitz filtered before one of these passages. As with the other viruses used in this study, there was no definite cytopathology.

Viruses in tissues other than lymph nodes. In addition to normal lymph nodes, tissue cultures of normal human skin and a variety of human neoplasms were used in these studies. Most cultures from these tissues contained fibroblastic as well as epidermal or neoplastic cells. In only a few of the cultures of cancer was it possible to be certain that neoplastic cells were growing. It was not possible to ascertain within what cell types the virus propagated. Bunyamwera virus was inoculated into a skin culture once and did not propagate. It probably did propagate in a culture of epidermoid carcinoma, as evidenced by a titer of $10^{-1.5}$ at 3 days after inoculation, at which time cultures were fixed. This culture was proved by back-implantation to contain cancer cells (see Fig. 31 to 33 in ref. 10), and appeared to be a pure culture of cancer cells. Hence it is highly probable that the virus was propagating in epidermoid cancer cells. Microscopically, necrosis of cells was more common in the virus-infected cultures than in the normal-mouse-brain controls, but many areas of healthy appearing carcinoma cells remained. Bunyamwera virus also propagated in cultures from reticulum sarcoma for 17 days. These cultures contained principally lymphoid (? lymphosarcoma) cells, but fibroblastic cells were also frequent. Egypt 101 virus persisted for 9 days in cultures of the same epidermoid carcinoma mentioned above. As with Bunyamwera virus there was suggestive but inconclusive evidence of cytopathology attributable to the virus. This virus also grew for 22 days in another epidermoid carcinoma culture and a renal adenocarcinoma culture, and for 24 days in a culture of rectal adenocarcinoma (see below). These cultures contained carcinoma cells, but also many fibroblasts. Egypt 101 virus also persisted for 34 days after inoculation into tissue cultures of melanoma. This virus was also demonstrated, without addition of stock virus, in cultures of malignant melanoma from 2 patients who had Egypt 101 virus infection with viremia. In each of these 3 cultures of melanoma the cells appeared fibroblastic, and there was no proof that melanoma cells were present. Egypt 101 virus was also inoculated into 3 cultures of human leukemic blood buffy coat. There was no evidence of virus propagation, but neither was there any evidence of cellular proliferation. Ilheus virus was inoculated into the same epidermoid carcinoma culture mentioned above and apparently propagated as evidenced by repeated recoveries over a period of 9 days. No definite cytopathology occurred.

Virus growth in cells from immune patient. Egypt 101 virus grew well for 24 days in tissue cultures of rectal adenocarcinoma (see Fig. 35 in reference 15) from an Egypt 101-immune patient, but disappeared promptly after addition of 0.2 ml of the same patient's immune serum. Addition of the

same serum to parallel cultures infected with Ilheus virus did not prevent continued propagation of the Ilheus virus. It has recently been reported that infectious canine hepatitis virus grows well in tissue cultures of kidneys from ICH-immune dogs(14).

Summary. 1. Bunyamwera virus was grown in roller-slide tissue cultures of human lymph nodes (fibroblastic type cells). Propagation of virus was demonstrated by increases in virus titers in short-term virus growth studies, by long persistence of virus in tissue cultures as contrasted with rapid disappearance in cell-free preparations, and by persistence of virus after serial passages had diluted the original virus inoculum far beyond its minimal infective dilution. 2. Similar but less extensive studies demonstrated the propagation in human fibroblast tissue cultures of West Nile, Egypt 101, Ilheus, and Br I viruses. 3. Bunyamwera virus showed changes interpreted as an adaptation phenomenon during serial passages in tissue cultures. These changes were reversed by serial intracerebral passage in mice. 4. None of these viruses caused appreciable cytopathology under these experimental conditions, even after prolonged growth in tissue culture. 5. Bunyamwera, Egypt 101, and Ilheus viruses were also grown in tissue cultures from various human cancers. It was not possible to determine whether virus was propagating in the cancer cells, or in non-neoplastic stromal cells. 6. Egypt 101 virus propagated in cells from an immune patient, but not in the presence of immune serum.

The authors are greatly indebted to Miss Priscilla J. Goettler, who performed much of the tissue culture technic involved in this study; and to Drs. Hilary Koprowski, Joseph Melnick, Alice Moore, and Kenneth Smithburn who supplied stock viruses.

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Specificity of Antineoplastic Phenomenon Induced in Mice by Carcinogenic Agents. (21045)

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Following the injection of methylcholanthrene into mice an antineoplastic (AN) factor appears in their spleens(1). This response appeared to be specific in that the AN factor was not engendered in homozygous mice through inoculations with phenanthrene and with cooking oil (the diluent), and it was not present in spleens from uninoculated mice. The AN phenomenon endured through a period that appeared to coincide with the latent period of carcinogenesis. This supposition was further supported by the absence

of the AN property in the spleens from tumorous mice.

This study has now been extended to observations on the chemical and genetic specificity of the AN phenomenon, using the tissue culture technic described in previous reports(1,2).

Methods. Exp. I. Induction of tumors. Groups of 28-30 young C3H mice* were each injected subcutaneously with 0.5 mg of a carcinogenic compound suspended in cooking oil. The compounds included methylcholanthrene (MCA)†; 1,2,5,6-dibenzanthracene (DBA)†; and 3,4-benzypyrene (BP).† These mice were observed until tumors appeared in the injected area. Two biweekly injections with BP and DBA were required to induce tumor formation. Exp. II. Studies on AN reactions with spleens. When tumors appeared in the mice of Exp. I, additional groups of 12-20 C3H mice and of CFW‡ mice were then injected with the same agents as noted above (0.5 mg in cooking oil subcutaneously). At 1 and 2 weeks following the latter injection, 4-5 mice from each group were sacrificed and their spleens were processed as follows: Each spleen was minced with scissors so as to reduce it to pieces approximately 1 mm in diameter. The minced tissue was washed 3 times in Hank's solution and suspended in 4 x the volume with Hank's solution and embryo extract (3 + 1). Each C3H mouse carrying a well established tumor was sacrificed by ether anesthesia. The tumor and spleen tissue from each animal was then individually processed in a manner similar to that described for the spleen. The processed tissues were explanted in coagulating chicken plasma on to the wall of a test tube. Tumor tissue was explanted on one side of the tube and spleen tissue on the opposite wall. One ml of nutrient fluid§ was added to each tube which was then tightly stoppered. The tissues were incubated at 35°C at a 5° angle, and rotated 6 times per hour. The tissues were studied in the following combinations: 1. Tumor plus "homologous" spleen from C3H mice. 2. Tumor and spleens from C3H mice, the latter inoculated 1 and 2 weeks previously with 1 of the carcinogenic compounds. 3. Tumor from C3H mice and spleens from CFW mice, the latter inoculated 1 and 2 weeks previously with a carcinogenic compound. The explants were observed microscopically at daily intervals and changes were recorded as to whether the explants were growing or not. No tissue combination was evaluated unless the spleen tissue showed evidence of viability. Control tubes contained tumor and spleen explants alone. The nutrient fluid was adjusted to optimal pH (7.2) with $NaHCO_3$ when needed.

Results. Tumors appeared in the mice at approximately 5 weeks following MCA inoculation and 12 weeks following inoculations with DBA and BP. The tumors were solid fibrosarcomas, subcutaneously localized and growing without evidence of metastasis.

The spleens from tumorous mice were frequently enlarged and engorged with blood, while those from more recently injected mice (Exp. II) were small, solid, and contained prominent Malpighian corpuscles.

Explants of tumor tissue proliferated as fibrocytes extending into the periphery, and forming a "halo" of cells around the original explant within 24-48 hours. Occasionally, the plasma clot was digested in one area thereby giving the appearance of a "signet ring" growth. Explants of spleen tissue expanded by quick migration of loose cells. Within 24-48 hours, lymphoid cells and fibrocytes surrounded the periphery of the explants. The type of cell around each explant was related to the presence of a Malpighian corpuscle in the explant, lymphoid cells predominating when such was present.

The fate of MCA-induced tumor explants with homologous spleen and with spleens from mice recently inoculated with MCA has been described(1). The tumors grew profusely with their homologous spleens; whereas, with the latter spleens noted above, the explants failed to grow. Recent inoculations of C3H mice with DBA and with BP engendered no such AN effect in their spleens against MCA-

* Jackson Memorial Laboratory, Bar Harbor, Me.

† Eastman Kodak Co.

‡ Carworth Farms, New City, N. Y.

§ Hank's solution; ox serum ultrafiltrate, embryo extract, penicillin, and streptomycin.

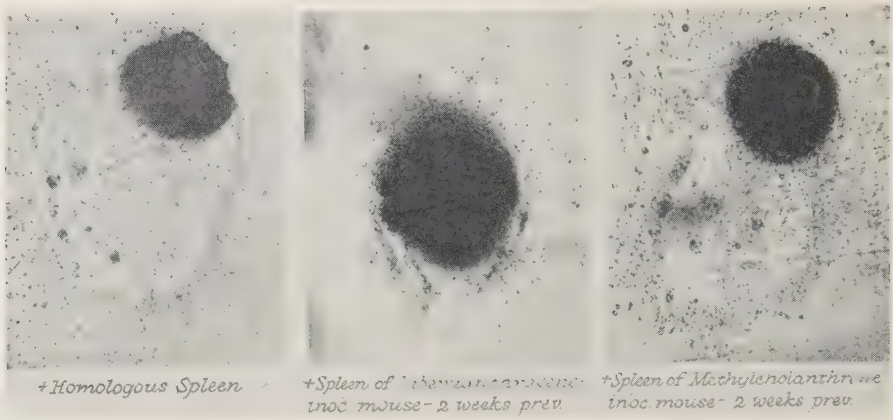

FIG. 1. Tissue cultures of 1,2,5,6-dibenzanthracene-induced mouse tumor—5 days. ×32.

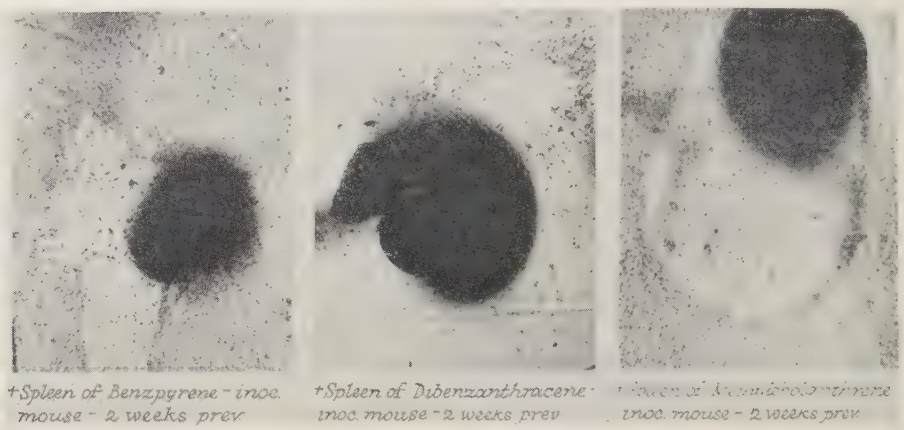

FIG. 2. Tissue cultures of 1,2,5,6-dibenzanthracene-induced mouse tumor—5 days. ×32.

induced tumors. Furthermore, CFW mice similarly inoculated with MCA failed to engender the AN factor in their spleens when cultured with MCA tumors from C3H mice (Table I).

DBA-induced tumor explants from C3H mice were attenuated and destroyed by spleens of C3H mice recently injected with DBA in contrast to the profuse tumor growth when explanted in combination with the homologous spleen (Fig. 1 and 2). The AN factor for DBA tumors in C3H mice was also absent from the spleens of C3H mice recently injected with MCA and with BP, as well as in CFW mice inoculated with all three of the carcinogenic agents (Table I).

BP-induced tumor explants from C3H mice were destroyed when explanted with spleen tissue from C3H mice recently inoculated with BP, whereas tumor growth was profuse with homologous spleen explants (Fig. 3). The AN phenomenon for BP-induced tumors was not demonstrable with spleen tissue from C3H and from CFW mice recently inoculated with DBA and with MCA.

When the growth of spleen tissue was incompatible with tumor growth, the cytopathogenic sequence was the same in all tumors. Usually the cells failed to grow out from the explant or if some growth was observed on the first day, the cells were fragmented and destroyed by the second day,

Discussion. We have here presented evidence indicating that the AN phenomenon is chemically and genetically specific. The findings support the previously reported observations that the response of mice to a recent injection with carcinogenic agent was a trans-

TABLE I. Effect of Spleen on Growth of Tumor Explants from C_3H Mice.

Tumor induced by:	Growth of tumor with homologous spleen	Effect of spleens from C3H and CFW mice inoculated 7-14 days previously with: MCA		BP		DBA	
		C3H	CFW	C3H	CFW	C3H	CFW
MCA*	13/13†	1/25‡	9/9	4/4	0	2/4	0
BP	11/11	8/8	0	5/20	6/6	11/12	0
DBA	9/9	5/6	0	5/5	0	0/6	2/2

* MCA, 20-Methylcholanthrene; BP, 3,4-Benzpyrene; DBA, 1,2,5,6-Dibenzanthracene.
† Number growing/tumors tested.
‡ Growth of tumor/spleens examined; 0, not tested.

ient AN effect(1). The specific AN phenomenon was demonstrable in a high percentage of mice inoculated with MCA, DBA and BP one and two weeks previously. This reaction has not yet been observed with spleen-tumor tissue combinations from the same mouse, regardless of the cause of the tumor (Table I). The initial AN response described above disappears with the appearance of the tumor. The intervening time, representing the latent period, might conceivably be altered by varying the quantity of inoculum, its route of inoculation, the age, and the genetic strain of mouse employed(3).

The spleen has been described by others as one of the most prolific of the antibody producing organs in the body(4,5). Antibody production may be the basis for the AN phenomenon observed; but the relationship between a carcinogenic agent and antibody response still needs clarification. The antibody response to a conventional antigenic stimulus can be detected 5 days after inoculation(6,7). Although we have not yet defined the exact mechanism responsible for the phenomenon, it becomes detectable 5 days after inoculation of the carcinogenic compounds.

When mice are inoculated with homotransplants of well established, transmissible tumors, the latent period is thereafter very short. Although this has not been studied extensively, some experiments with the technics described above indicate that the AN response following tumor transplantation disappeared 8 days after the tumor was inoculated. The mechanism whereby a host is thus rendered susceptible to a homologous tumor may represent an accelerated development of what eventually evolves through the action of a carcinogenic agent.

The chemical and genetic specificity of the AN phenomenon conforms with the reports of others(8,9) regarding the susceptibility of a specific species and strain for the optimal transmission of a tumor: the genetic strain

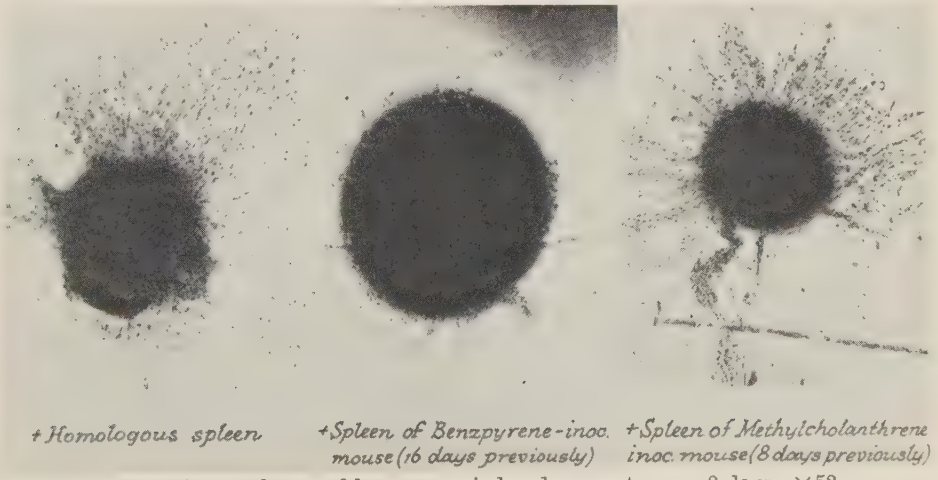

Fig. 3. Tissue cultures of benzpyrene-induced mouse tumor—2 days. ×52.

from which a tumor emerged providing the most fertile soil for its subsequent propagation.

Summary. When mice were inoculated with 3 carcinogenic compounds, they developed a transient antineoplastic (AN) response which was demonstrable by tissue culture technic. This AN phenomenon appeared to be chemically and genetically specific. The duration of the AN response to the 3 agents may represent the latent period of carcinogenesis, since the AN phenomenon disappeared when the tumor emerged. The interpretation of the AN phenomenon on an immunological basis is discussed.

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Topical Protection against Inflammation by a Preceding Local Inflammation in Adrenalectomized Rats.* (21046)

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Acquisition of topical protection against inflammatory reaction to blood-borne irritants such as egg-white or dextran has been shown in the rat-paw after local pre-irritation with various irritants(1). Further, it has been demonstrated that thermally pre-irritated tissues display topical protection against a subsequent strong thermal irritation leading to inflammation and necrosis in normal tissues (2). One of the authors reviewed earlier data on acquired topical protection against tissue injury and studied mechanisms underlying the acquisition of topical protection against blood-borne irritants and against thermal and X-irradiation injury(3). Recently, an interesting contribution concerning this subject was made by Arteta(4). He observed that adrenalectomy prior to local pre-irritation (peptone injection in the rat-paw) prevented the development of topical protection; thus, the pre-irritated paw displayed apparently undiminished inflammatory reaction to egg-white intraperitoneally injected 24 hours after pre-irritation. However, topical protection could be demonstrated in adrenalectomized rats, if cortisone was intraperitoneally injected 1 hour after the local pre-irritation, the egg-white being given 24 hours later. On the basis of this finding, Arteta advanced the theory that a topical pre-irritation evokes a General Alarm Reaction, and that "the steroids of suprarenal source which are produced during this reaction may move from the blood to the inflamed tissue due to the increase of capillary permeability which takes place in the inflamed area, accumulating there and modifying the tissue's conditions and the degree of resistance of the capillary wall." The preferential accumulation of antiphlogistic steroids in inflamed areas seems to be an intriguing suggestion in connection with the mode of action of these steroids. Some hesitation was felt, however, in accepting this view as an explanation of the mechanism underlying acquired topical protection. Therefore, further investigations were made on the problem of acquired topical protection to intraperitoneally injected egg-white and to thermal

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† Fellow of the Canadian Life Insurance Officers Assn.

TABLE I. Acquisition of Topical Protection in Adrenalectomized Rats.

Groups*	1st irritation Inj. in right hind paw	2nd irritation Intraper. inj. 24 hr later	% degree of protection in pre-irritated right paw (mean ± stand. error)
I†	Peptone 4%, .25 ml	Egg-white .5 ml	64.7 ± 8.2
II†	*Idem*	" 20% 1.0	94.6 ± 3.1
III†	Dextran .12%, .2 ml	" .5	96.6 ± 2.5
IV†	*Idem*	" 20% 1.0	95.3 ± 2.5
	Heating right hind paw	Heating both hind paws, 48 hr later	
V‡	47°C, 2 min. 15 sec.	49°C, 2 min.	77.4 ± 1.9

* 6 animals in each group.
† Adrenalectomy 6 hr prior to 1st irritation.
‡ " 10 days " " " " .

injury in adrenalectomized rats.

Methods. Male Sprague-Dawley rats weighing between 100 and 120 g were used. Bilateral adrenalectomy was performed under ether anesthesia and subsequently the animals were allowed 1% saline as drinking fluid and Purina Fox Chow *ad libitum*. Peptone and dextran (Macrodex) were injected beneath the plantar aponeurosis of the right hind paw. Heating [immersion of the paw(s) into water under compression of the blood vessels], evaluation of the degree of inflammatory reaction, and assessment of the protection in the pre-irritated paw were made as described earlier(2).

Results. In corroboration of Arteta's findings(4), whose experimental conditions were adhered to in Group I, only negligible topical protection was observed in some of the adrenalectomized animals in this group (Table I). If, however, the amount of egg-white injected intraperitoneally for the second irritation was reduced (Group II), or if dextran was used for topical pre-irritation (Groups III and IV and Fig. 1A), the pre-irritated right paw displayed maximal topical protection against the hyperergic egg-white reaction in all adrenalectomized animals. In rats adrenalectomized 10 days prior to a thermal pre-irritation of the right hind paw (Group V and Fig. 1B), marked topical protection was observed against subsequent stronger thermal injury.

On the basis of these results, it is not very likely that the acquisition of topical protection depends upon extravasation of antiphlogistic corticosteroids. Since adrenalectomized rats become more susceptible to substances which cause anaphylactoid inflammation [dextran(5,6), globin(6)], one possible alternative to the interpretation given by Arteta (4) could be the assumption that intraperi-

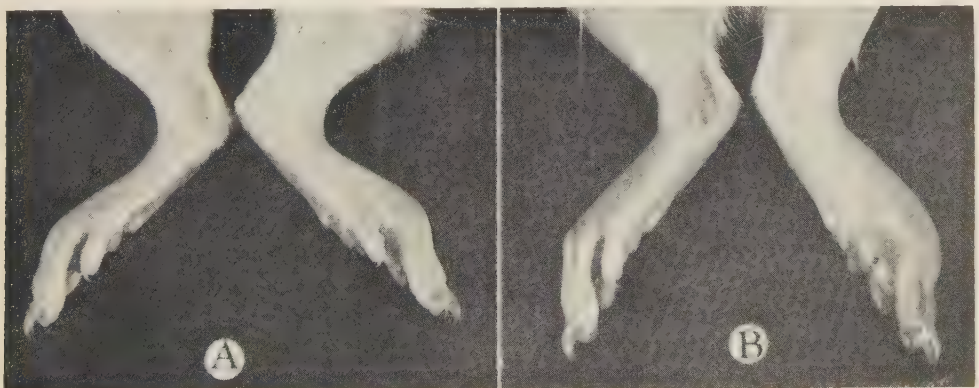

FIG. 1. Acquisition of topical protection in adrenalectomized rats. Left: Animal of Group III, 2 hr after intraperitoneal injection of egg-white; pre-irritated right paw protected; (adrenalectomy 6 hr prior to pre-irritation). Right: Animal of Group V, 14 hr after heating of both paws; pre-irritated right paw protected; (adrenalectomy 10 days prior to pre-irritation).

toneal injection of undiluted egg-white leads to a *maximal* increase in capillary permeability in the paws, and that, in this situation, the protective effect of certain inflammatory substances used for pre-irritation might become less evident.

Summary. 1. Topical protection against hyperergic inflammation by a preceding local inflammation, and topical protection against thermal injury by a preceding local thermal irritation can be demonstrated even in rats adrenalectomized before pre-irritation. 2. Acquired topical protection, therefore, is not likely to depend upon the topical extravasation of antiphlogistic corticosteroids into pre-inflamed areas.

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Susceptibility of Mice to Infection with the Mahoney Strain of Type 1 Poliomyelitis Virus.* (21047)

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A line of the Mahoney strain Type 1 poliomyelitis virus has been adapted by Li and Schaeffer(1) to produce intraspinal infection in mice. This strain has been used to demonstrate neutralizing antibodies in serum specimens(2), and also to demonstrate resistance upon challenge of mice immunized with Mahoney virus(3). Since the intraspinal inoculation is a rather traumatizing procedure and the non-specific response due to trauma, depending on the skill of the operator, is considerable, a line adapted to the intracerebral route might therefore be advantageous. Furthermore, increasing adaptation might eventually result in a line pathogenic to mice following peripheral inoculation.

It is the purpose of this report to give details of the procedure that resulted in a line of Mahoney virus pathogenic via the intracerebral route. This strain will also induce paralysis in a small proportion of mice following intravenous inoculation.

Materials and methods. Virus. Mahoney strain virus adapted for intraspinal infection of mice was kindly provided by Drs. Li and Schaeffer as 10% cord suspension representative of the 35th mouse passage. This material was used for intraspinal titration in mice and as the inoculum for tissue cultures. Four-week-old Swiss albino mice, CFW strain, were employed as routine. Tissue culture fluids from criss-cross passages 3, 4, 6 and 7 were identified as Type 1 poliomyelitis virus: a) by neutralization test in tissue cultures using homotypic antibody for poliomyelitis types,

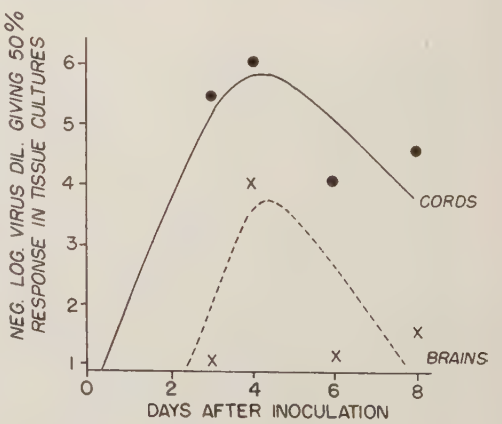

FIG. 1. Distribution of virus in brains and cords of paralyzed mice at various intervals after intravenous inoculation of the 4th criss-cross passage fluid.

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1-2-3; b) by intracerebral challenge of mice immunized by inactivated Type 1 virus. The technic for adaptation consisted of serial criss-cross passages between tissue cultures and mice by the injection intracerebrally, or intravenously of tissue culture fluids into mice and by recovery in tissue culture of virus from cord tissue of paralyzed animals. The technics for criss-cross passage, the preparation of tissue cultures, and the preparation of the cord suspensions were described previously (4).

Undiluted tissue culture fluid from the first passage was tested in mice by injection intracerebrally, 0.03 ml, or intravenously, 0.5 ml. For successive passages, the inoculum consisted of cord from the first mouse paralyzed from intracerebral injection when intravenous injection had proved ineffective within 5 days from injection. Tissue culture fluid from the 4th criss-cross passage was used as inoculum to study the distribution of virus in brain and cord of mice developing paralysis following intravenous inoculation. Tissue suspensions of cord and brain from paralyzed mice were prepared and the supernatant fluid after centrifugation was titrated in 10-fold dilution steps for virus activity in tissue cultures using 3 cultures per level. Mice receiving the unadapted Mahoney strain intravenously did not develop paralysis. Their cords and brains were treated in the same manner and used for comparison.

Results. The titer of virus in the cord was more than 100-fold higher than in the brain of the same animals. The highest virus concentration in the cord was found in mice paralyzed early after inoculation, the titer was less in the 6- and 8-day specimens. These findings are in good agreement with those of Herrarte and Pearson(5) who followed the virus concentration in the cords and brains of mice after intracerebral inoculation with Type 2 poliomyelitis virus. No virus was recovered from mice inoculated with the unadapted Mahoney virus.

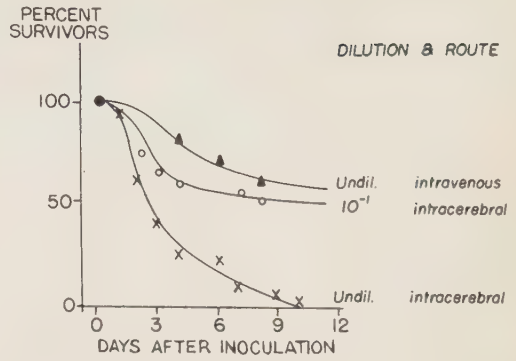

FIG. 2. Response-time relationship in mice inoculated with tissue culture fluid from the 7th criss-cross passage.

Fig. 2 shows the time-response relationship for the intracerebral and intravenous inoculation of tissue culture fluid taken from the 7th criss-cross passage. Twenty mice were used for each 10-fold dilution. 50% survival time estimated from this graph is about 2.5 days for the group inoculated intracerebrally with undiluted tissue culture fluid, the incubation period is 2 to 6 days, sometimes as short as one day but rarely animals responded later than the 10th day.

TABLE I. Potency of Tissue Culture Fluids Harvested from Various Criss-Cross Passages Tested in Tissue Cultures and in Mice Inoculated by Different Routes.

	Tissue cultures				Mice								
					Intraspinal					Intracerebral			Intravenous
Doses (ml)	.25				.025					.025			.5
Neg log dilutions	5	6	7	Log ID_{50}/ml	2	3	4	5	Log ID_{50}/ml	0	1	Log ID_{50}/ml	0
35th M.P.					5/8	4/8	0/8	0/8	4.1				
1st C.C.P.	3/5	0/5	0/5	5.6	5/10	1/10	1/10	0/10	3.6	1/10	0/10		0/10
2nd	4/5	1/5	0/5	6.0						5/20	0/20	0.75	
3rd	2/5	1/5	0/5	5.6	9/10	6/10	1/10	0/10	4.5	9/20	1/20	1.5	5/12
4th	4/5	3/5	0/5	6.5						15/20	4/20	2.0	4/20
5th	5/5	3/5	1/5	6.8	10/10	5/10	1/10	0/10	4.6	12/20	4/20	1.75	3/20
6th	5/5	1/5	3/5	6.8	10/10	7/10	3/10	0/10	5.0	16/20	6/20	2.0	2/20
7th	5/5	5/5	3/5	7.5	10/10	10/10	4/10	2/10	5.5	20/20	8/20	2.3	8/20
8th	5/5	5/5	5/5	>7.5						20/20	12/20	2.5	

TABLE II. Neutralization Test of Type 1 Passage Virus in Mice and Tissue Cultures. Entries Are Proportions Responding.

Neutralization test in	Virus passage	Approx. ID_{50}	Immune monkey serum			Normal monkey serum
			Type 1 Brunhilde	Type 2 Lansing	Type 3 Saukett	
Mice (I.C.)	7th C.C.P.	32	2/20	18/20	20/20	20/20
Tissue culture	" "	1000	0/3	3/3	3/3	3/3
		100	0/3	3/3	3/3	3/3

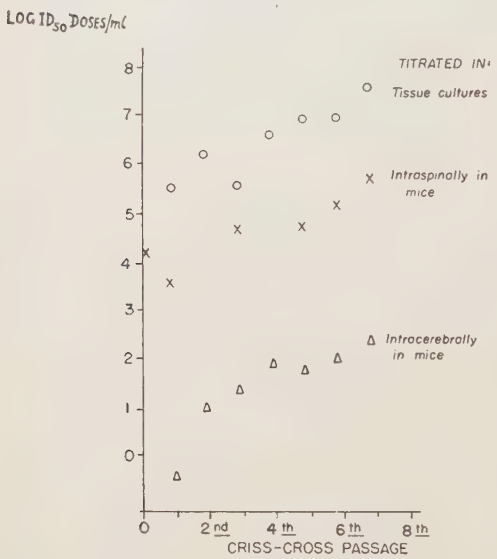

FIG. 3. The potency at various passage levels of the Mahoney strain in tissue cultures, and in mice inoculated by different routes.

Table I contains data on the potency of tissue culture fluids from the 1st to the 8th criss-cross passage when tested in mice inoculated by various routes as well as in tissue cultures. The ID_{50}/ml taken from this table are plotted in Fig. 3. These data suggest that during the course of serial criss-cross passages the potency of the tissue culture fluids increased for mice as well as for tissue cultures.

Evidence that this strain retained its immunological identity during the course of these adaptation experiments is presented in Table II. The cytopathogenic effect in tissue cultures and the infectivity in mice is neutralized by an anti-Brunhilde monkey immune serum. Equally potent immune sera representing Type 2 and 3 poliomyelitis virus as well as normal monkey serum had no neutralizing effect.

Discussion. Evidence is presented for the successful adaptation of the Mahoney strain to the intracerebral route in mice. The same material induces paralysis in a small proportion of mice following intravenous injection. The rise in infectivity following criss-cross passages is suggested by the data. However, it is questionable whether this is the only factor involved in the adaptation. So far only 2 out of 6 Type 1 fluids having the same potency for tissue cultures as the 6th criss-cross passage have induced paralysis in a small number of mice inoculated intraspinally.

In the course of these experiments the strain under investigation acquired the ability to invade the spinal cord, and high concentration of virus in the cord is associated with the development of paralysis. The dissociation in infectivity for the intraspinal and intracerebral route of inoculation is striking. A well adapted Lansing strain will be almost equally potent by those two routes. Therefore, it seems conceivable that the Mahoney strain is not yet adapted to its full mouse pathogenicity or, in this respect, may behave differently from the Lansing strain.

Summary. Criss-cross passages between mice and tissue cultures resulted in the adaptation of Type 1 (Mahoney) poliomyelitis virus to the intracerebral route in mice.

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Influence of Post-Irradiation Medication with Antibiotic JM-57h on Survival Time in Mice.* (21048)

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Investigations have shown that post-irradiation medication with various antibiotics has increased survival through reduction of the infection which follows acute whole body exposure to ionizing radiation(1-9). Miller *et al.*(9) have pointed out that not all antibiotics are effective because they could not control infections with Pseudomonas, Proteus or Salmonella. Recently Stavely(10) stated that the broad spectrum antibiotic JM-57h was effective against the above organisms so we have used it in an attempt to increase survival time in irradiated mice.

Experimental. Male CF-1 strain mice, weighing an average of 24 g each, were arranged in groups of 20 animals each. Except during irradiation, the animals were maintained in an air-conditioned room at 72 ± 5°F and fed a diet of Rockland pellets supplemented weekly with additional vit. A and D. The animals received daily intramuscular injections of 0.36 mg of antibiotic JM-57h† or saline in a total volume of 0.1 ml following the dosage schedules given in Table I and continuing until 90-100% mortality had been attained. An additional non-irradiated antibiotic control group was medicated for 18 days. The 550 r radiation dose was administered from above and below the mice with two 250 KVP Picker Industrial Units operating simultaneously. The technical factors were: 250 KVP; 15 MA; FOD 100 cm; filters, 0.21 mm Cu inherent, 0.5 mm Cu parabolic, and 1.0 mm AL; HVL 2.02; size of field—total body; r/min. measured in air 17.19. Both units were calibrated prior to each experiment with a Victoreen thimble r-meter. The animals were restrained in a plastic cage similar to the one previously described for guinea pigs(11). The results obtained were analyzed statistically by the method of Litchfield(12).

Results. Upon the basis of the data in Table I, it is evident that, in general, antibiotic JM-57h did not significantly increase the total number of survivors although it had some effect on survival time. Medication throughout the experimental period (Group 6) resulted in a significant increase in the ST_{50} compared to the saline controls (Group 5). It is also apparent that the animals must be medicated during the first post-irradiation week if any benefit whatsoever is to be obtained (compare Groups 2 and 6 with the balance of the medicated groups). From the infection viewpoint, the first post-irradiation week is critical because the leukocytes are disappearing at a rapid rate while a bacteremia is developing. Vincent and Veomett (13) found that a massive bacteremia from Proteus and Pseudomonas organisms begins during this time interval and Miller *et al.*(9) made similar observations in the CF-1 mouse. Thus it would appear that after irradiation injury, antibiotic medication with an agent which has a more specific effect on Proteus and Pseudomonas organisms, must be started on the first post-irradiation day and continued for at least 15 days or until the natural body defense mechanisms have recovered their function.

The deaths in the antibiotic treated animals cannot be ascribed to the drug, although the dose used was 0.1 of its LD_{50} (highest dose tolerated chronicly), because there were no deaths or untoward reactions in Group 9. Furthermore, these animals gained an average of 2 g during the experimental period.

Summary. Antibiotic JM-57h, a wide spectrum agent, has been used to combat post-irradiation infection without significantly increasing the total number of survivors. In

* This paper is based on the work performed under Contract between the Atomic Energy Commission and the University of California at Los Angeles.

† The authors wish to thank Dr. Homer E. Stavely of Commercial Solvents Corp. for the supply of antibiotics JM-57h used in this investigation.

TABLE I. Survival Time in Antibiotic-Treated Irradiated Mice. 9 groups.

Treatment	Days	ST_{50},* range in days	Slope, range	Total mortality %	Total mortality Day
Saline	1– 7	11.4 (10.8–12.0)	1.12 (1.08–1.16)	95	13
Antibiotic	1– 7	12.2 (11.4–13.1)	1.18 (1.12–1.24)	90	16
Saline	8–15	12.0 (11.0–13.1)	1.22 (1.15–1.3)	90	17
Antibiotic	8–15	11.7 (11.2–12.2)	1.11 (1.07–1.14)	100	18
Saline	1–18	10.7 (10.3–11.1)	1.08 (1.06–1.11)	100	13
Antibiotic	1–18	12.2 (11.7–12.8)	1.11 (1.07–1.15)	95	16
Saline	8–18	11.0 (10.3–11.8)	1.16 (1.11–1.22)	100	15
Antibiotic	8–18	10.0 (9.1–10.9)	1.23 (1.15–1.31)	100	14
Non-irradiated controls, antibiotic	1–18	0	0	0	18

* ST_{50} = day on which 50% of animals were still alive. All values at odds of 19/20.

the group medicated daily an increase in the ST_{50} day was noted. The results obtained indicate that medication must be started during the first post-irradiation week before massive bacteremia occurs, otherwise such therapy will not be beneficial. At a dose of 0.1 of its LD_{50}, antibiotic JM-57h was well tolerated and produced no fatalities or untoward reactions in CF-1 strain mice.

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Therapeutic Interference and Potentiation between Subtilin and Gold Sodium Thiomalate in Spirochetal and Streptococcal Infections.* (21049)

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The interference phenomenon in protozoan infections was widely studied by the school of Ehrlich and Morgenroth to explore the mechanism of chemotherapeutic action. Morgenroth and Rosenthal discovered that the trypanocidal activity of antimonyltartrate is inhibited by hexatantalate, a non-trypanocidal agent, which still induces drug resistance to antimonials(1). Browning—a pupil of Ehrlich—and Gulbransen, reported in 1922 the first case of interference between 2 effective drugs, by inhibiting the activity of acriflavin with pararosaniline, on a trypanosome strain resistant to the latter drug(2). Schnitzer(3) established the phenomenon in normal trypanosome strains and with his coworkers extended these findings to arsenicals and antimonials(4,5) and just recently reported(6) the original Browning-Gulbransen interference in the experimental *Trichomonas vaginalis* infection of mice. In bacterial infections the interference phenomenon between therapeutically effective drugs appeared only after the discovery of the antibiotics. We may refer to the studies of Price, Randall, Welch, and Chandler(7) and of Jawetz, Gunnison, Speck, and Coleman(8). Characteristic of the difficulties of interpretation of the experimental findings is the statement of Bliss, Warth, and Long(9): "Aureomycin and chloromycetin are usually reported as antagonistic toward penicillin (6 references quoted), but several authors have pointed out that the effect varies with the concentration of the agents (3 references quoted) and Spicer got different results with different bacteria."

The present study represents an example of antagonism between 2 chemotherapeutic agents which extends to both their antispirochetal and antibacterial activity.

TABLE I. Interference between Subtilin and Myochrysin in the *β hemolytic Streptococcus* (C-203) Infection of Mice. Subtilin was injected subcutaneously on the abdomen, myochrysin immediately after on the back, at the time of inoculation with 100 MLD = 0.5 cc 10^{-7} culture broth.

Exp.	Subtilin + myochrysin (mg/kg)		Survived total
I	—	—	0/10
	.5–1.0	—	8/20
	2	—	6/10
	2	5	7/10
	2	10	10/10
	2	20	4/10
	2	40–80	20/20
II	—	—	0/10
	4	—	6/10
	4	5	10/10
	4	10	6/10
	4	20	6/10
	4	40	5/10
	4	80	10/10
	—	20	0/10
	—	40	0/10
	—	80	3/10
	—	100	10/10

Materials and methods. Mice were infected with the African strain of *Borrelia duttoni* and with *B. obermeieri.* For the *Streptococcus hem.* infection the C-203 strain was used; the intraperitoneal inoculum consisted of ca. 100 min. lethal doses. Drug treatment was given by the subcutaneous route and the min. effective doses were accurately established. In the case of combined treatment, when not otherwise specified, the 2 drugs, subtilin and gold sodium thiomalate (myochrysin), were injected at the same time, always at different subcutaneous sites.

Results. 1. *Streptococcus hemolyticus infection.* The average therapeutic dose of subtilin is 1.8 mg/kg, of myochrysine, 50 mg/kg. Two characteristic interference experiments are presented in Table I. There is an indication that low, sub-therapeutic myochrysin doses (5-10 mg/kg) reinforce the chemotherapeutic action of subtilin, but a

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TABLE II. Interference between Subtilin and Myochrysin in the β *hemolytic Streptococcus* Infection of Mice.

Subtilin + myochrysin (mg/kg)		No. mice	Survivors, %
1.25–1.5	—	50	44
1.25–1.5	12.5–25	70	20
2.5	—	70	84
"	6.25–12.5	80	65
"	25	70	47
"	50	20	90
"	100	20	95
—	25	40	12.5
—	50	10	60
—	100	40	92.5

higher myochrysin dose (20-40 mg/kg) establishes interference. A further dose increase of the gold compound dose causes a synergistic action. Table II summarizes all other results and shows on a larger series of animals that the optimal interfering dose of myochrysin against 2.5 mg/kg subtilin is about 25 mg/kg, *i.e.*, 1/3-1/2 of the therapeutic dose. With a lower subtilin dose, 1.25-1.5 mg/kg, the interference phenomenon was very definite at the 12.5 mg/kg myochrysin level. Thus, it would seem that within certain limits, the higher the dose used, the higher is the dose of the other drug of the pair required for interference.

2. *Borrelia duttoni infection.* Darkfield parasite counts were made 3, 5-6, 8, 22, and 40-44 hours after drug treatment. The dose of subtilin resulting in a temporary reduction of the spirochetes is 8-10 mg/kg, the average clearing dose, 16 mg/kg. The dose of myochrysine which clears the majority of the mice within 24 hours is 12.5 mg/kg. In one group of experiments 8 mg/kg subtilin alone induced a temporary (3-8 hours) parasite decrease of different magnitude in 18 of 31 (= 58%) mice; 22 hours after treatment, the spirochetes returned to the control level in 27/31 mice, and were only slightly reduced in the remaining 4. In the parallel group treated with 12.5 mg/kg myochrysine alone there was a significant early (5-8 hours) decrease of parasites in 8/21 mice; 22 hours after treatment, 20/21 were completely cleared and the remaining mouse showed an 80% reduction in the parasite count. The combined treatment with the above doses resulted in an initial parasite decrease in 17/21 mice (= 81%); 22 hours after treatment, 13/21 were completely cleared, 4 were reduced and 3 were at the control level. Thus, the delayed effect of the combined treatment seems lower than that of myochrysin alone.

Experiments involving 89 mice treated with doses of 4-8 mg/kg subtilin and 5-6 mg/kg myochrysin alone and in combination gave the impression that the combined treatment might perhaps have increased somewhat the early subtilin effect and possibly accelerated the clearing action of myochrysin without influencing the number of animals cleared. (The dose of myochrysin used cleared ca. half of the mice within only 48 hours.)

The difficulty in comparing the variables of the parasitemia curves in these experiments, *i.e.*, incidence, degree, time of appearance and duration of the reduction, did not lead to a definite conclusion on the effects of the combined treatment, but to the clue that under certain conditions interference might be possible. In fact, by varying time intervals between the administration of the 2 drugs and dosages, we finally obtained quite clear cut cases of reciprocal interference, as for instance those presented in Tables III and IV. The early action of subtilin and the delayed effect of myochrysin could be decreased by combined treatment. It would appear that in order to obtain the interference phenomenon it is necessary to keep an interval of at least one hour between drug administration, and one of the drugs has to be maintained below its therapeutic level.

On the other hand, by increasing the doses of both drugs to therapeutic levels or above, a synergistic action seems to prevail. For instance, combined treatment with 16 mg/kg subtilin and 12.5 mg/kg myochrysin doses give an effect which can be mainly visualized as the superposition of the early subtilin and the delayed (22 hours) myochrysin actions. However, the immediate action of subtilin can also be increased using for combination higher, 25-50 mg/kg, myochrysin doses as it would appear from the following results. Subtilin doses of 8 mg/kg alone—adding up our experiments—gave complete clearing

TABLE III. Interference between Subtilin and Myochrysin in the *Borrelia duttoni* Infection of Mice.

Exp. No.	1st treatment, mg/kg	Interval, hr	2nd treatment, mg/kg	No. spirochetes/25 fields ..... hr after treatment: Before	3	5–6	22	40–44
165F	Myo 6		—	25	110	130	60	3
	"		—	30	75	110	80	1
	"		—	25	100	1000	250	400
	"		—	25	100	125	20	0
	Myo 6	3	Subt 8	50	75*	25	2	35
	"	"	"	25	170*	20	50	0
	"	"	"	50	100*	7	60	0
	"	"	"	25	150*	0	0	0
	"	"	"	25	125*	25	0	175
	—		Subt 8	30	200*	0	0	10
	—		"	75	175*	0	50	25
	—		"	50	200*	0	10	125
	—		"	50	175*	0	0	450
	—		"	30	175*	0	0	80
167F	Control†	—	—	75	150	400	1000	1750
	Myo 5‡		—	75	125	150	75	50
	Myo 5	1	Subt 4	75	150	200	22	15
	"	"	"	50	175	200	20	18
	"	"	"	50	100	175	25	5
	"	"	"	100	75	100	150	10
	—		Subt 4	100	125	2	50	75
	—		"	75	0	4	60	80
	—		"	75	0	5	180	200
	—		"	100	125	160	250	275

* Time of 2nd treatment. † Avg of 3 mice. ‡ Avg of 4 mice.

within 3 and 5 hours in 19% of 78 mice. Myochrysin doses of 25-50 mg/kg alone did not clear any of 6 mice during this period, but only after 20 hours, while the combination treatment cleared all 6 mice within 5 hours.

3. *Borrelia obermeieri infection.* This infection is more sensitive to subtilin and less to myochrysin than the duttoni strain. The reducing dose is 1.5 mg/kg subtilin and 25 mg/kg myochrysin (in 24 hours), the clearing doses, 2.5 and 37.5 mg/kg, respectively(10).

Table V summarizes the results of the combined treatment. Both possibilities, interference and potentiation, were encountered in this infection too. It would also appear that the interfering combinations are formed around the range of effective dosages, which once surpassed, lead to synergism. It should be noted that the effects of the combination treatment may vary according to the observation period considered.

Discussion. From a quantitative viewpoint, the interference between myochrysin and subtilin in the streptococcal infection is in line with previous observations on this phenomenon. There is an optimal interfering dose which is overcome by an excess of the drugs(5,11).

In retrospect, we understand the reasons which made difficult the establishment of the interference in the spirochetal infection: (a) First, the effect may vary at different time intervals and therefore a careful comparison of the whole curve of parasitemia is required. (b) Combined treatment with sub-therapeutic dosages remains mostly ineffective. (c) Therapeutic dosages of *both* drugs generally reinforce each other's action, thus at this level the interference no longer appears. (d) The interference is most marked when a sub-therapeutic dose of one drug is followed by a therapeutic dose of the other. (e) The subtilin effect is somewhat variable and therefore the same dose may or may not provoke interference according to whether it was subeffective or effective. Consequently, the interference phenomenon between the 2 drugs studied is contained within very narrow margins.

TABLE IV. Interference between Subtilin and Myochrysin in *Borrelia duttoni* Infection of Mice.

Exp. No.	1st treatment, mg/kg	Interval, hr	2nd treatment, mg/kg	No. spirochetes per 25 fields ..... after treatment							
				Before	3 hr	5 hr	8 hr	1 day	2 days	3-4 days	5-8 days
102F	Myo 12		—	25	75	50	15	0	0	0	25
	"		—	40	80	50	19	0	0	0	30
	"		—	25	75	50	10	0	0	0	3
	—		Subt 10	25	150*	1	0	4	40	0	5
	—		"	40	125*	2	5	10	150	2	3
	—		"	25	125*	10	19	8	200	0	1
	Myo 12	3	"	25	80*	25	10	0	0	0	2
	"	"	"	25	50*	50	3	0	0	0	1
	"	"	"	25	75*	50	19	0	0	0	3
	"	"	Subt 16	25	75*	4	0	0	0	0	3
	"	"	"	30	100*	1	0	0	0	0	2
	"	"	"	25	100*	12	7	0	0	0	3
	—		"	25	125*	0	0	2	15	5	25
	—		"	25	110*	0	0	0	0	10	25
	—		"	25	125*	1	0	1	10	6	4
112F	Subt 4	3	Myo 12.5	20	60*	40	3	3	75	350	
	"	"	"	30	75*	30	125	7	110	250	
	"	"	"	30	80*	60	150	50	80	350	
	"	"	"	35	110*	60	200	75	110	180	
	—		"	25	170*	100	75	0	0	0	
	—		"	35	175*	80	150	0	0	0	
	—		"	40	150*	90	250	0	0	0	
	—		"	25	180*	75	200	0	0	0	
118F	Subt 1	3	Myo 12.5	25	80*	75		2	0	1	
	"	"	"	25	100*	50		3	25	7	
	"	"	"	25	75*	60		0	0	2	
	Subt 4	"	"	18	25*	20			Died		
	"	"	"	25	25*	25		1	0	0	
	"	"	"	25	25*	25		1	0	0	
	—		"	25	75*	75		0	0	0	
	—		"	25	100*	75		0	0	0	
	—		"	25	80*	100		0	0	0	

* Time of treatment (second).

On the basis of the foregoing it can be explained also why the *absolute* doses required to obtain the phenomenon depend on the sensitivity of the spirochetal strain to the interfering drugs.

The lack of additive action of sub-therapeutic doses of myochrysin and subtilin may be interpreted in various ways: it could be the result of an interference in the "undetectable" range, but it could also indicate that the therapeutic action depends on independent cell receptors each of which has to be attacked up to a certain threshold effect before the 2 lesions can integrate each other to influence the vitality and the reproductive rate of the spirochetes. We are inclined to believe that in both infections myochrysin and subtilin act by mechanisms which are not identical. The BAL reversibility of myochrysin in both infections, in contrast to that of subtilin(12), gives a certain support to our hypothesis. It has been said(13) that the antibacterial action of subtilin is due to its surface activity, "damaging the cell membrane, rather than by blocking a particular metabolic step," while for the Au compound its thiol binding capacity is generally accepted as the mode of action.

In a previous case of interference between antibiotics and arsenoxide we have analyzed the arguments in favor of the interpretation that the interference involves other systems than the final receptor sensitive to the biochemical lesion produced by the drugs(11). The same considerations should be valid in the present case. It may be noteworthy from this standpoint that among the antispirochetal agents assumed to act by similar

TABLE V. Effects of Combined Subtilin and Myochrysin Treatment in the *Borrelia obermeieri* Infection of Mice. 8 mice in each series.

Exp. No.	mg/kg		Hr after treatment			
	Subtilin	Myochrysin	2-3	5-5½	22-23	29
9	1.0	—	++	++	+	±
	—	15.0	0	0	0	±
	—	20.0	0	0	±	+
	1.0	15.0	Ind	Ind	Ind	Syn
	1.0	20.0	Intf	Sl. intf	Ind	Sl. syn
	1.5	—	+++	+++	+++	++
	1.5	15.0	Ind	Ind	Syn	Syn
	1.5	20.0	Sl. intf	Ind	Syn	Syn
7	1.5	—	+++	+++	++	++
	—	12.5	0	±	0	0
	1.5	12.5	Ind	Ind	Sl. syn	Sl. syn
	3.0	—	+++	+++	++	++
	3.0	12.5	Ind	Syn	Syn	Syn

Effect of single treatment: 0 = ineffective, ± = slight effect, + = moderate effect, ++ and +++ = marked effects.
Effect of combination: Ind = indifference, Syn = synergism, Intf = interference.

mechanisms on the basis of their *dithiol reversibility* only one (chloromycetin) interferes with arsenoxide, while the others (penicillin, bacitracin) remain indifferent(14). Further, other *non dithiol reversible* antibiotics, such as aureomycin and terramycin, interfere also with arsenoxide(12).

The necessity for a certain interval between treatments to determine interference depends most likely on the period required for a sufficient uptake of the interfering drug to block the absorption-fixation and/or the passage of the second drug to the final receptor.

In a broad sense, this differentiation between systems responsible for interference and for the chemotherapeutic lesion is parallel to the "primäre" and "sekundäre giftbindende Kerne" in the frame of the chemoceptor theory of Ehrlich(15).

Summary. 1. Myochrysin interfered with the therapeutic activity of subtilin in the experimental β hemolytic streptococcus infection of mice. 2. The myochrysin dose required for interference increased with the amount of subtilin used (it varied between 12.5-40 mg/kg). 3. In the *Borrelia duttoni* infection of mice, subtherapeutic doses of one drug interfered with the therapeutic action of the other, while therapeutic doses of both drugs acted synergistically. 4. Analogously, in the *Borrelia obermeieri* infection, both interference and synergism were noted. 5. The effect of the combined treatment in the spirochetal infection may vary at different time intervals following treatment.

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Application of Induced Pulmonary Arterial Collateral Circulation as Collateral Supply to the Heart.* (21050)

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Although the existence of an extracoronary blood supply to the heart has long been known (1), this has usually proved insufficient to prevent infarction after occlusion of a major coronary artery. Numerous attempts have been made to augment the collateral circulation, by inducing the formation of granulation tissue within the pericardial sac(2,3), by causing the heart to adhere to the pectoral muscle or omentum(4), by implanting an internal mammary artery into the myocardium (5,6), by creating a shunt between the coronary sinus and a systemic artery(7,8), and by pericardiopneumonopexy. Following the last named procedure Lezius(9) demonstrated the development of direct connections between the coronary and pulmonary artery. It is difficult to understand how the desaturated blood in the pulmonary artery could be of great benefit to the myocardium, even assuming that a flow from lung to heart would occur upon occlusion of a coronary artery.

Cardiopneumonopexy after ligation of a large pulmonary artery would seem a more logical procedure, for it has been abundantly demonstrated that such a lung soon receives an abundant collateral supply from the aorta (10-12).

Method. The left pulmonary artery was ligated in a series of dogs, and adhesions between this lung and the anterior and left lateral aspects of the heart were assured by cauterizing the surfaces to be apposed with a silver nitrate stick, and suturing them together through a surgically created defect in the pericardium. At the end of the experiment vinylite plastic corrosion casts of the coronary and pulmonary vessels and bronchial tree were made(13).

Results. In 5 of 7 animals sacrificed 9 to 20 weeks after the operation just described abundant transpleural vessels were found to connect the coronary and bronchopulmonary arterial circulations (Fig. 1). Five percent vinylite in acetone, introduced into the left coronary artery, easily entered the greatly enlarged bronchial arterial collateral vessels of the lung. In most animals many of the pulmonary arteries were also injected, since they remain patent distally beyond the ligature, and now communicate with the bronchial arteries by means of large anastomoses in contrast with their state in the normal dog.

Discussion. In this way the myocardium now becomes connected with the aorta in two ways: 1) by the coronary arteries through their ostia in the sinuses of Valsalva, and 2) by means of the expanded bronchial arteries, with the thoracic aorta. It is probable that initially the blood flow is from the coronary arteries to the lung. Whether this can be reversed, to the benefit of the myocardium, after interruption of the coronary arterial circulation remains to be determined.

Summary. When adhesions are created between the heart and a lung the pulmonary artery of which has been ligated, abundant large connections develop between the coronary arteries and vastly expanded bronchial arteries. The efficiency of these connecting vessels as collateral blood supply to the heart is under investigation.

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FIG. 1. Anterior view of vinylite plastic cast of heart and lungs of dog 9 weeks after ligation of left pulmonary artery and cardiopneumonopexy. Large arteries (arrows) pass via adhesions to connect coronary arteries and enlarged bronchial collateral arteries of the left lung. The trachea is seen to descend in the midline, above. Anteriorly of the trachea is an accessory bronchial artery (black). The right auricle overlies the distal end of the trachea.

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